

Oral Cells and Tissues

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Chapter 1

Early Tooth Development



Teeth are formed from oral epithelium, in the form of a dental lamina, and neural crest ectomesenchyme of the maxillary and mandibular processes (Fig 1-1). The oral epithelium contributes the enamel component, and the ectomesenchyme contributes the dentin and cementum components of the fully formed tooth. Although the initiating events that trigger downgrowth of the oral epithelium to form a dental lamina are incompletely understood, it is known that neural crest ectomesenchyme is necessary.^{1,2} Early reciprocal inductive interactions between the oral epithelium and the underlying ectomesenchyme, and subsequent interactions between the enamel organ and dental papilla, coordinate the sequential events of tooth development.³⁻⁵

Efforts to understand the instructional signals that originate in each of these interacting tissues have been ongoing for more than 50 years.^{6,7} Most investigations have been performed with dental tissues obtained from embryonic mice and rats or with the continuously growing incisor teeth of adult mice and rats. Organ culture techniques have been perfected to study the growth of dental tissues in chemically defined media, to observe the results of various epithelial-mesenchymal combinations, and to examine the effects of various growth factors on odontogenesis. Thus, nearly all current insight into the regulatory mechanisms of tooth development has come from studies of animal models, often from tooth buds grown in organ culture.

This chapter contains a discussion of the initiation of tooth formation and the histodifferentiation of the enamel organ and dental papilla. Subsequent chapters will examine the cytodifferentiation of dentin- and enamel-forming cells and the secretion and mineralization of their respective matrices.

Role of the Neural Crest

Early in embryogenesis, soon after the neural tube forms by invagination of the overlying ectoderm, migratory pluripotent neuroepithelial cells, the neural crest cells, migrate from the dorsal midline region of the neural tube.⁸ In exiting from the neural tube, neural crest cells lose their epithelioid characteristics and assume a mesenchymal phenotype capable of directed cell migration. Cranial neural crest cells invade the developing branchial arches and, in a series of reciprocal inductive interactions with early oral epithelium, form tooth primordia (Figs 1-1 and 1-2). When the movement of dye-injected neural crest cells was traced in organ cultures of developing dental arches, it was shown that neural crest cells from the posterior midbrain, and to a lesser extent from the anterior hindbrain, form dental ectomesenchyme.⁹ The failure of neural crest ectomesenchymal cells to migrate normally to appropriate sites during craniofacial development leads to serious developmental defects, including the absence of

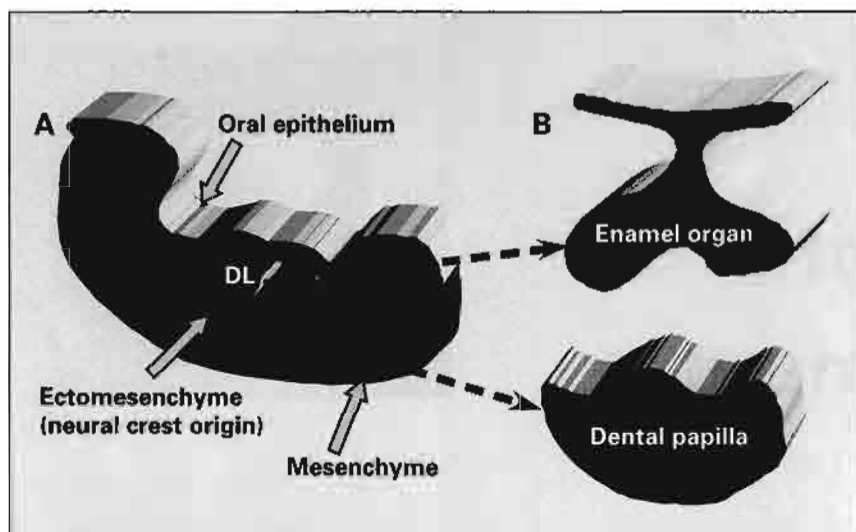


Fig 1-1 Stages in the development of a tooth bud. (A) Oral epithelium and the underlying ectomesenchyme and mesenchyme during the development of the dental lamina (DL). (B) The enamel organ arises from a genetically determined site of the dental lamina by cell proliferation. The dental papilla develops from ectomesenchymal cells of neural crest origin.

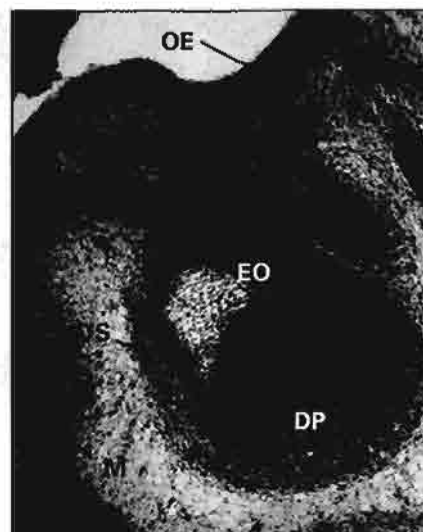


Fig 1-2 Histologic section of a developing tooth at early bell stage. (DL) Dental lamina; (DP) dental papilla; (DS) dental sac; (EO) enamel organ; (M) mesenchyme; (OE) oral epithelium; (SL) successional lamina. (Hematoxylin-eosin stain. Original magnification $\times 220$.)

teeth (anodontia) and underdeveloped jawbones (micrognathia).

Subsets of cranial neural crest cells give rise to chondrocytes, osteoblasts, periodontal ligament fibroblasts, cementoblasts, and odontoblasts. Final phenotype differentiation is regulated by interaction of the ectomesenchymal cells with extrinsic factors, such as growth factors, and substrate adhesion molecules in the local microenvironment.¹⁰ It has been suggested that there may be separate populations of neural crest cells for each tooth type. The molecular code for each tooth type appears to reside in specific sets of homeobox genes.^{11,12}

Development of the Dental Lamina, Enamel Organ, and Dental Papilla

The first evidence of tooth formation in humans is observed as a thickening of the oral epithelium in the mandibular, maxillary, and medial nasal processes in the 1-month-old fetus (Figs 1-3 to 1-5). It has been suggested that the zone of epithelial thickening (the dental plate or placode) contains the genetic deter-

minants for the initiating signals that regulate the number and position of the future tooth buds. Experiments with epithelial-mesenchymal tissue recombination have shown that early-stage oral epithelium is capable of inducing tooth development in non-oral ectomesenchyme.¹³⁻¹⁵ When non-oral epithelium is used in the recombination, only bone and cartilage form in the ectomesenchyme. Mouse oral epithelium has been shown to induce biochemical markers of early tooth development in chick oral ectomesenchyme, a tissue thought to have lost its ability to form teeth.¹⁶ The results of these studies suggest that the oral ectoderm contains instructional signals for tooth development and perhaps the prepattern for the entire dentition. Weiss et al¹⁷ suggested that a very early signaling system (prior to neural crest migration) involving *Shh* and *Pax6* genes might form the basis of epithelial patterning mechanisms for tooth development.

Formation of the dental lamina

At a slightly later stage of development (11- to 14-mm embryos), the epithelium invaginates into the underlying mesenchyme to form the dental lamina. This process begins in the distal (molar) region and later

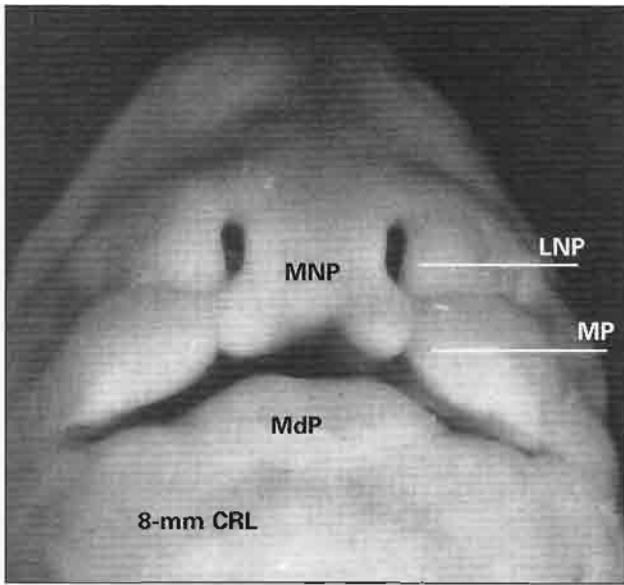


Fig 1-3 Facial region of a human embryo. (LNP) Lateral nasal process; (MNP) medial nasal process; (MP) maxillary process; (MdP) mandibular process; (CRL) crown-rump length. (Adapted from Ooe⁷⁴ with permission.)

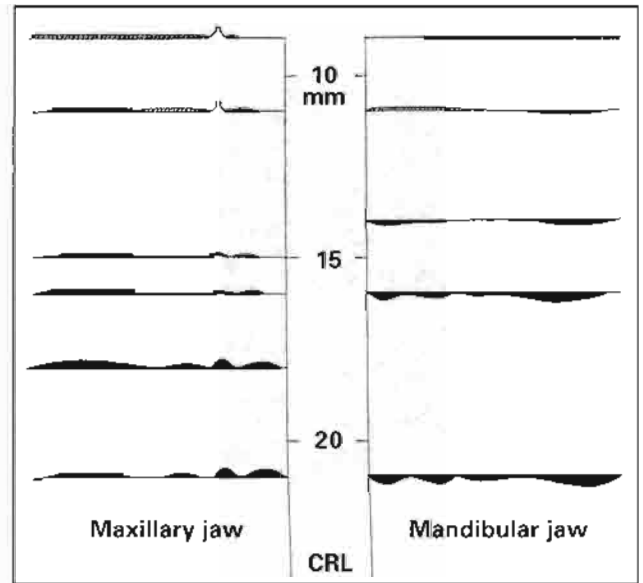
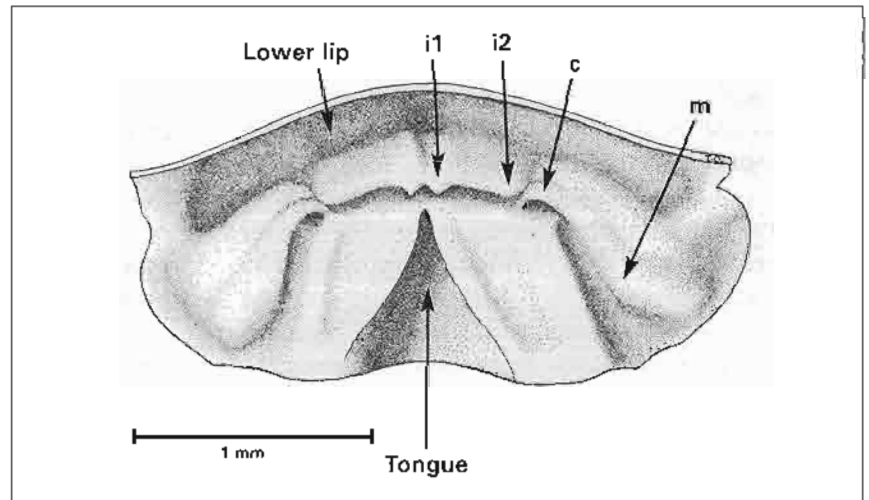


Fig 1-4 Degree of oral epithelial thickening in various human embryos ranging from 10- to 20-mm crown-rump length (CRL). Note the undulating character of the undersurface of the epithelium. (Adapted from Ooe⁷⁴ with permission.)

Fig 1-5 Model of the reconstructed oral epithelium of the mandible in a 16-mm human embryo. The "swellings" correspond to the sites of early development of the future primary central incisor (i1), lateral incisor (i2), canine (c), and molar (m) tooth buds. (Adapted from Ooe⁷⁴ with permission.)



in the midline. In 15- to 20-mm human embryos, the dental lamina shows signs of additional differential growth, reflecting the determination of incisor, canine, and molar domains (see Figs 1-4 and 1-5). Deep notches in the dental lamina are present between the incisor and canine domains, especially in the mandible. Continued site-specific enlargement of the dental lamina, along with condensation of neural crest ectomesenchyme, gives rise to the individual tooth buds.

Role of homeobox genes

Recent studies of the role of homeobox genes indicate that the expression of these genes in ectomesenchymal tissues may control the development and ultimate shape of the tooth.^{11,18-20} Homeobox genes constitute a large family of genes that specify correct positioning of body parts during embryonic development. These genes are implicated in determining axial patterns, such as the anteroposterior development of limbs. All members of this family

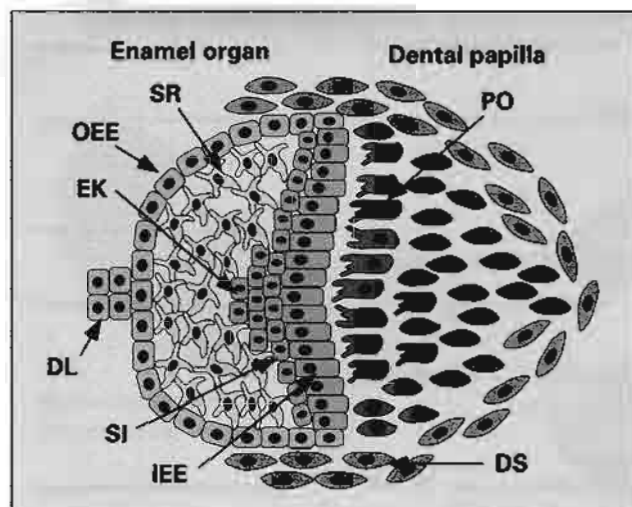


Fig 1-6 Enamel organ and dental papilla. The outer enamel epithelium (OEE) forms the convex surface of the enamel organ and is separated from adjacent dental sac (DS) cells and general mesenchyme (not shown) by a basement membrane. The stellate reticulum (SR) lies between the OEE and the stratum intermedium (SI). The SI cells are closely juxtaposed to the cells of the inner enamel epithelium (IEE). The enamel knot (EK) represents a small group of nondividing cells near the IEE. The IEE is separated from the preodontoblasts (PO) of the dental papilla by a basement membrane (see Fig 1-8). (DL) Remnant of the dental lamina.

share a common code for a 60-amino acid DNA-binding sequence (the homeodomain) that allows the protein to act as a gene regulatory factor. Homeobox genes (*Dlx*, *Pax*, *Msx*, etc) are widely expressed in embryonic craniofacial tissues. Whiting²¹ has reviewed their role in normal development as well as the developmental defects that result from mutations.

Studies of tooth development in mice that have mutant homeobox genes support the idea that regional expression of various homeobox genes may provide the positional information for the type of tooth to be formed.¹¹ The results of these studies indicate that mutations in *Dlx1* and *Dlx2* genes prevent maxillary molar development but have no negative effect on maxillary incisor development. Incisor development is regulated by *Msx1* and *Msx2* homeobox genes. Thus, according to Thomas et al,¹¹ the odontogenic pattern (ie, tooth type and position in the arch) is determined by early regional and restricted expression of various combinations of homeobox genes. Once the tooth buds are formed, the homeobox genes are activated in a more generalized pattern. The presence of *Msx1* is required for progression of molar tooth development beyond the bud stage.^{20,22}

Karg et al²³ described the localization of the homeobox gene, *S₈* (*Prx2*), in the dental papillae of developing mouse incisor and molar tooth buds. Because the highest level of *S₈* expression occurs during the growth of the dental papilla, it was suggested that *S₈* might take part in regulating the overall growth of the developing tooth. At the cap stage of tooth development, epithelial growth centers (enamel knots) regu-

late the cuspal outline of the developing tooth by coordinating cell proliferation within the enamel organ and dental papilla through the secretion of growth factors.^{24,25}

Progress in research on gene expression in tooth development can be found on the Internet at <http://bite-it.helsinki.fi>.²⁶

Histogenesis of the tooth

The enamel organ develops by proliferation of cells in the dental lamina. The adjacent ectomesenchymal cells continue to proliferate and concentrate to form the dental papilla and dental sac (see Fig 1-2). During this coordinated growth, various growth factors and regulatory proteins are exchanged between the epithelium and ectomesenchyme.

During the early stage of tooth development, the enamel organ, shaped like a cap, is superimposed over a condensation of ectomesenchymal cells (Figs 1-2, 1-6, and 1-7a). At the cap stage, the enamel organ is subdivided into four regions: the outer enamel epithelium (OEE), the stellate reticulum (SR), the stratum intermedium (SI), and the inner enamel epithelium (IEE) (see Fig 1-6).²⁷⁻³⁰ Later in development, the enamel organ is bell shaped, encompassing a well-defined dental papilla along its concave internal surface (Fig 1-7b).

The cells of the OEE are cuboidal and separated from the adjacent dental sac ectomesenchyme by a basement membrane. Along their concave surface, they contact the star-shaped cells of the SR. The cells of the SR are separated by wide intercellular spaces. Adjacent SR cells remain in contact via long

Figs 1-7a and 1-7b Three-dimensional reconstructions of enamel organs made from serial sections of human embryos. Dental papilla and mesenchyme not shown. (Adapted from Ooe⁷⁴ with permission.)

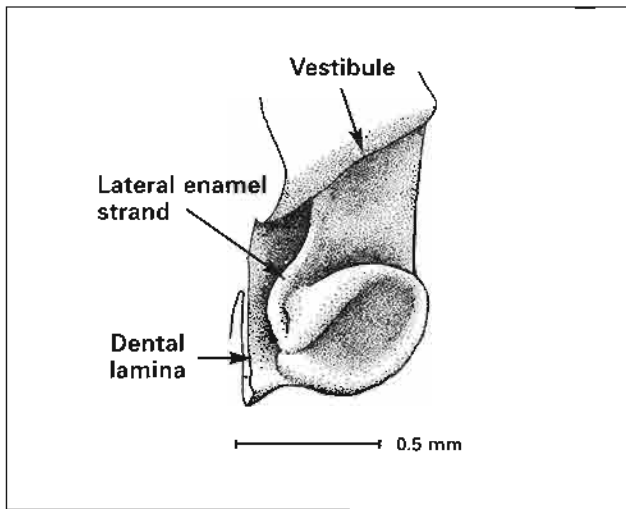


Fig 1-7a Cap stage.

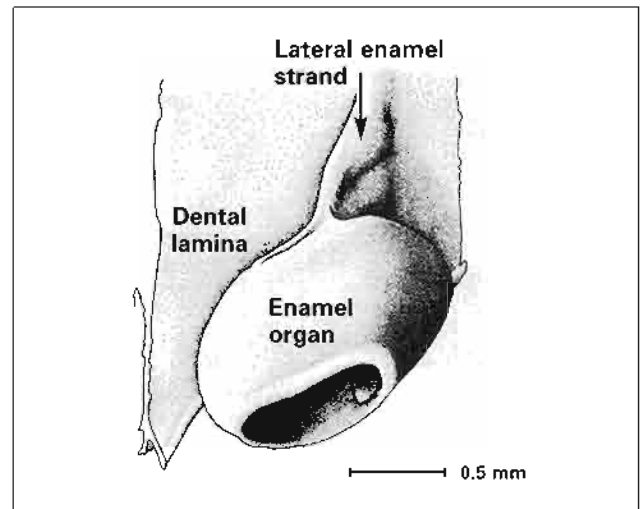


Fig 1-7b Bell stage.

cytoplasmic folds joined by numerous desmosomes and gap junctions (see Fig 1-6). The intercellular spaces of the SR contain hyaluronan and chondroitin sulfates that bind large amounts of water.³¹ The SR retains its hydrated state until the initiation of enamel formation; thereafter, the SR and the OEE differentiate into the papillary layer (described in chapter 3).

The SI consists of one or two layers of low cuboidal cells situated between the SR and the IEE (see Fig 1-6). A clearly defined SI is established between the SR and the IEE just prior to the differentiation of the ameloblasts. The cells of the SI and IEE express similar enzyme patterns, suggesting that both cell types have common metabolic functions.

The cells of the IEE are juxtaposed to the ectomesenchymal cells (preodontoblasts) of the dental papilla (Figs 1-6 and 1-8). The basement membrane beneath the IEE consists of a basal lamina densa and many aperiodic fibrils (see Fig 1-8). The nature of these fibrils and their significance in odontoblast differentiation are discussed in chapter 2.

Cytodifferentiation of odontoblasts and ameloblasts starts at the tip of the future cusps. Under the influence of stimuli originating from the IEE, the preodontoblasts begin differentiation. In turn, they stimulate the cells of the IEE to undergo differentiation to form a single layer of enamel matrix-secreting cells, the ameloblasts. Preodontoblasts reach maturity as secretory odontoblasts before the preameloblasts mature into secretory ameloblasts. Regulatory control of

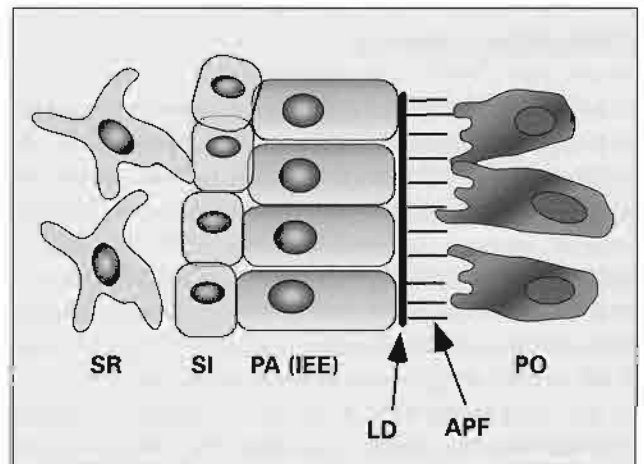


Fig 1-8 Role of basement membrane components at the junction between the preameloblast (PA) of the inner enamel epithelium (IEE) and the adjacent preodontoblast (PO). A basement membrane consisting of a lamina densa (LD) and aperiodic fibrils (APF) separates the two tissues. The POs extend cell processes toward the APFs. (SR) Stellate reticulum; (SI) stratum intermedium.

cell proliferation and the differentiation of ameloblasts and odontoblasts is provided in part by complex sequential interactions involving cell membrane receptors, growth factors, and/or matrix molecules concentrated in the IEE basal lamina. Recent research has begun to define regulatory signals in tooth development at the level of gene activation.^{32,33}

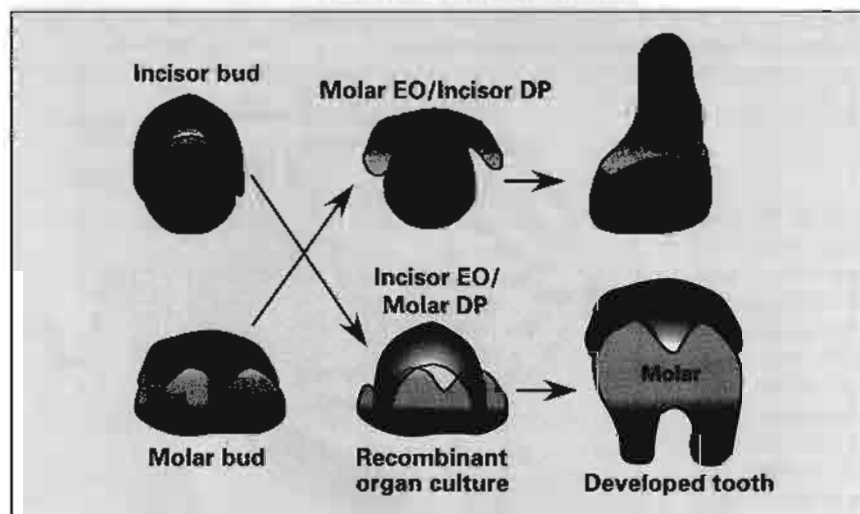


Fig 1-9 Control of tooth shape by the dental papilla (DP). Dissociation of the enamel organ from the dental papilla by low calcium and trypsin digestion of the basement membrane makes it possible to study the development of various recombinations. Organ cultures of recombined tissues demonstrate the controlling influence of ectomesenchyme (dental papilla) on final tooth form. (EO) Enamel organ. (Based on the findings of Kollar and Baird.^{34,35})

Epithelial-Ectomesenchymal Morphogenetic Regulation of Odontogenesis

During the 1930s, the science of experimental embryology developed hand-in-hand with advances in organ culture technology. It soon became possible to grow whole and disassociated tooth buds in vitro. Enamel organs, when separated from the dental papillae by trypsin digestion of the basement membrane, were cultured alone or in various recombination with non-oral mesenchymal tissues (Figs 1-9 and 1-10). Isolated cap stage enamel organ, grown either in vivo as a transplant or in vitro in an organ culture system, failed to produce ameloblasts. Dental papilla cells failed to differentiate into odontoblasts unless grown in contact with the enamel organ. These studies established the need for contact between the epithelium (enamel organ) and the ectomesenchyme (dental papilla) as a preliminary condition for the differentiation of ameloblasts and odontoblasts. It was also observed that the dental papilla, once established, controlled the shape of the tooth and gained the ability to direct the differentiation of overlying epithelium (see Figs 1-9 and 1-10).³⁴⁻³⁶

When it was discovered that the odontogenic inductive interaction could take place across a thin, porous filter, the search for diffusible soluble factors responsible for inducing the differentiation of ameloblasts and odontoblasts became the mission of several dental researchers. In the late 1960s and early 1970s, as the science of molecular biology was

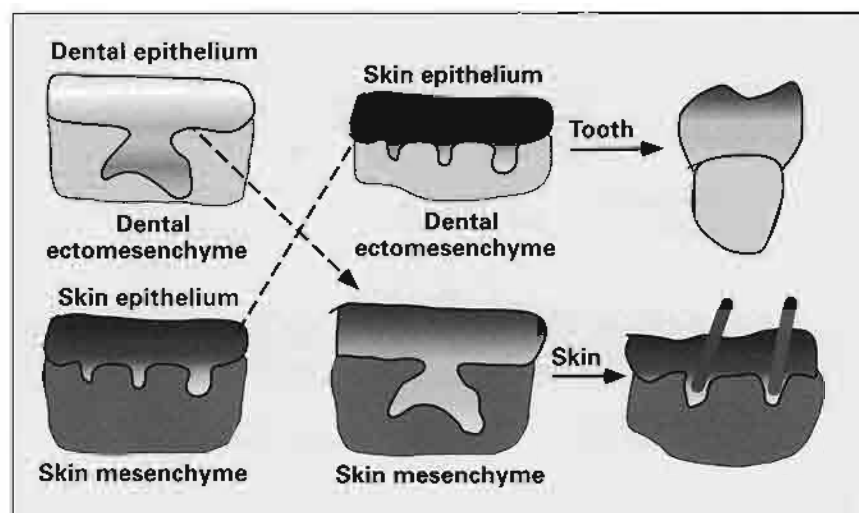
being developed, it was speculated that the transfer of informational messenger ribonucleic acid (mRNA) across the basement membrane might control the differentiation of odontogenic cells. In the 1970s, electron microscopic studies showed that cell-to-cell contacts were formed between preodontoblasts and preameloblasts during the cytodifferentiation stage of tooth development. It was proposed that such contacts might provide informational clues responsible for initiating differentiation. Because additional evidence in support of these hypotheses was not forthcoming, attention was directed to the extracellular matrix as a potential communication link between the enamel organ and the dental papilla. This premise was supported by the apparent importance of the basal lamina during odontoblast differentiation.

Role of matrix-mediated signaling

The discovery that enamel organs expressed amelogenin transcripts when cultured on a basement membrane gel, but not when grown on a laminin-coated filter, reinforced the concept that cell-matrix interactions had a permissive effect on gene transcription during tooth development. Research was soon focused on the interactions of cell membrane receptors with specific extracellular matrix ligands as important signaling events that might regulate odontogenic cell differentiation. These findings led Ruch et al to state:

Experimental data demonstrate that dental histomorphogenesis and cytodifferentiation are con-

Fig 1-10 Inductive action of mesenchyme on epithelial differentiation. Organ cultures of dental epithelium recombined with skin mesenchyme develop skin epidermis, complete with skin appendages. When skin epithelium is cultured in contact with dental mesenchyme, a tooth is formed, complete with enamel organ. These results demonstrate the inductive influence of mesenchyme on epithelium. (Based on the findings of Kollar.³⁶)



trolled by an alternative flux of information circulating between ectomesodermal and epithelial cells. They are matrix-mediated signals. The basement membrane is a dynamic, asymmetric interface demonstrating compositional and conformational modulations. The spatial pattern and timing of these changes result from specific activities of adjacent cells.⁴

Based on numerous *in vitro* experiments, Ruch et al⁴ proposed that basement membrane modifications are causally related to successive steps of odontogenesis. The following are the essential points of this hypothesis:

1. Time- and space-specific information is encoded in the basement membrane constituents.
2. This information is read by cell membrane receptor molecules of adjacent cells.
3. Receptor-ligand interactions act on the cytoskeleton and/or cytoplasmic enzymes, which subsequently influence transcriptional and posttranscriptional events.

To date, fibronectin, fibronectin receptors, tenascin, and syndecan have been implicated as participants in matrix-mediated signaling during odontogenesis.

The distribution of cell adhesion molecules and substrate adhesion molecules as potential control factors in tooth development has been a subject of increasing interest. Syndecan, a proteoglycan cell adhesion molecule located in the cell membrane, is

expressed prior to tooth formation in the ectomesenchymal cells that underlie the dental epithelium.³⁷ Tenascin, a large substrate adhesion molecule, is expressed in the ectomesenchyme during the down-growth of the dental lamina and during the subsequent condensation of the dental papilla.³⁸ It has been proposed that the binding of membrane-bound syndecan molecules to extracellular tenascin molecules is responsible for the condensation of the ectomesenchymal cells.^{37,39}

An alternative explanation is that tenascin interferes with cell-to-fibronectin attachment, leading to decreased migration of the ectomesenchymal cells, causing them to aggregate in the form of the dental papilla. Adhesion of fibroblasts is weaker to fibronectin than to tenascin.⁴⁰ It has also been shown that when cells express syndecan they have a reduced ability to invade a collagen gel. Thus, the appearance of syndecan on the cell surface of ectomesenchymal cells may have a direct, negative effect on their ability to migrate, thereby causing them to form aggregates, such as the dental papilla.

Tissue separation and recombination studies have demonstrated that the expression of syndecan and tenascin in tooth ectomesenchyme is induced during specific epithelial-mesenchymal interactions.⁵ *In situ* hybridization studies indicate that mRNA for tenascin is expressed in high amounts in cells of the inner enamel epithelium and the preodontoblasts. Redundant pathways regulating cell condensation are undoubtedly present, because tooth development has been shown to proceed normally in mice lacking tenascin expression.⁴¹

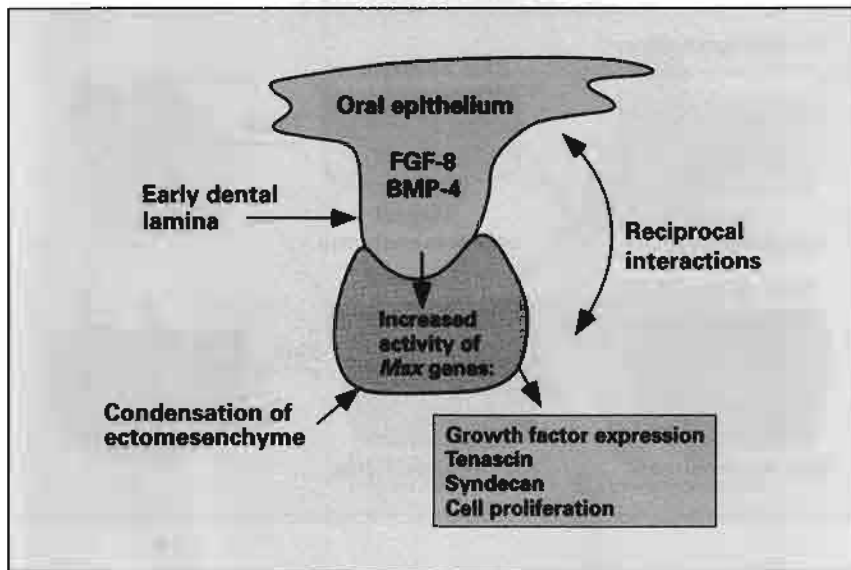


Fig 1-11 Proposed model of molecular mechanisms in early tooth bud development, illustrating the role of bone morphogenetic protein 4 (BMP-4) in activating *Msx* gene expression and a cascade of differentiation within the underlying ectomesenchyme. With the activation of *Msx* genes, the inductive potential is transferred to the dental ectomesenchyme. Reciprocal interactions involving signaling growth factors, matrix molecules, and cell surface receptors regulate cell differentiation. Enamel knot signaling centers appear in the enamel organ prior to cusp formation. (FGF-8) Fibroblast growth factor 8. (Based on the findings of Vainio et al.^{38,46})

Role of growth factors

Advances in organ culture technique have made it possible to grow developing teeth in chemically defined culture media. Yamada and coinvestigators⁴² demonstrated that explants of developing teeth could undergo complete cell differentiation and matrix mineralization in a chemically defined medium. They concluded that autocrine and paracrine factors coordinate the sequence of cellular differentiation events during tooth development. This stimulated the search for diffusible growth and regulatory factors that might be involved in odontogenesis.

Using chemically defined culture media, Chai et al⁴³ showed that tooth size and rate of development are regulated in part by transforming growth factor β 2 (TGF- β 2). When antisense oligonucleotides against TGF- β 2 are added to tooth organ cultures, development is accelerated and the tooth buds grow larger than controls.⁴³ Addition of exogenous TGF- β 2 reverses the effect of antisense nucleotides, leading to normal growth.

The advent of powerful molecular biologic approaches marked the beginning of a new era by discovery of the regulatory role of growth factors in dental morphogenesis. Thesleff and colleagues^{5,33,44,45} have reviewed recent advances in this area of developmental biology. The earliest growth factor signal

emanating from the presumptive dental lamina epithelium is bone morphogenetic protein 4 (BMP-4)^{5,46} (Fig 1-11). Epithelial cells make BMP-4 until the cap stage, when the production of BMP-4 shifts to the condensed ectomesenchymal cells. Soon thereafter, a new bone morphogenetic protein (BMP-2) appears in the epithelial cells. These shifts in BMP expression may account for the transfer of instructional activity from the epithelium to the dental papilla ectomesenchyme at the cap stage.

It has been proposed that BMP-4 activates *Msx* genes in the adjacent ectomesenchymal cells⁴⁶ (see Fig 1-11). The *Msx* genes are "muscle segment" members of the homeobox genes (regulators of segmentation) that have been implicated as regulators of the mesiodistal axis of tooth bud placement. *Msx* gene products are believed to be transcription activators that regulate the expression of BMPs, syndecan, and peptide growth factors in the condensing ectomesenchyme (see Fig 1-11). At the bell stage, *Msx2* is active in secondary enamel knots (EKs) and in the dental papilla.

Transcription products of *Msx1* function during later stages of tooth development, possibly regulating the differentiation of ameloblasts and odontoblasts.²⁰ Animals that lack the *Msx1* gene fail to develop teeth.²²

An especially important discovery was the identification of the enamel knot as a signaling center within

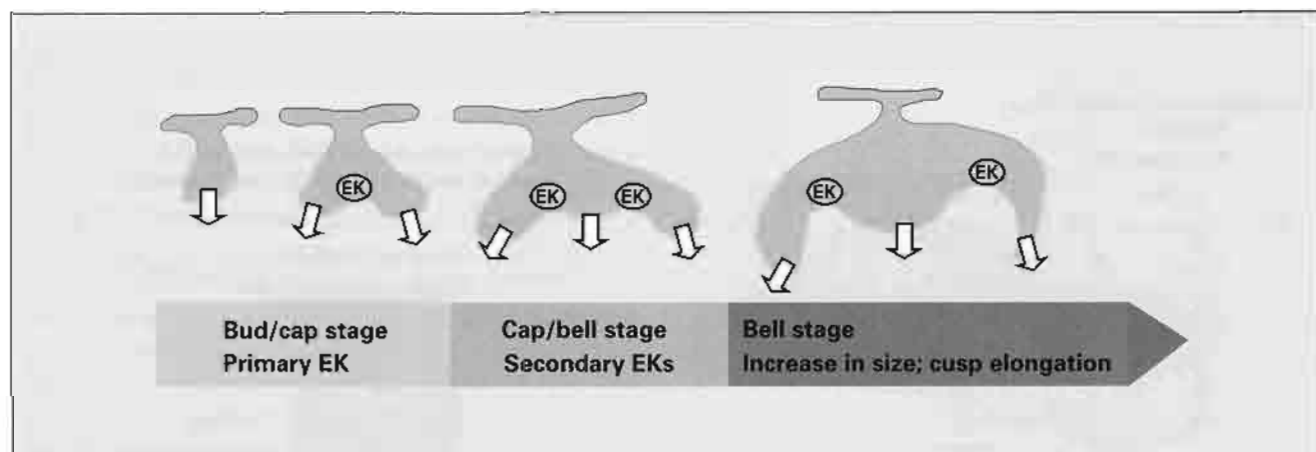


Fig 1-12 Possible role of the enamel knot (EK) in cusp formation. (arrows) Direction of growth. During the cap stage, the epithelium grows laterally around the dental mesenchyme. A single EK coordinates the development of the early cap stage. In multicusped teeth, secondary EKs are formed over future cusps to coordinate development during the late cap stage to the bell stage. (Adapted from Jernvall et al²⁴ with permission.)

the enamel organ.^{24,25,47} The enamel knot, a component of the enamel organ previously believed to be unimportant, has achieved prominence as a potential regulatory center of cell proliferation involved in cusp formation. The EK is a small group of closely packed, nondividing cells located adjacent to the IEE, and, in a single-cusped tooth, close to the center of the enamel organ (Figs 1-6 and 1-12).

The earliest sign of EK formation appears to be the localized expression of BMP-2 and BMP-7 in epithelial cells of the dental lamina and enamel organ. In situ hybridization techniques demonstrate that EK cells produce fibroblast growth factor 4 (FGF-4), several bone morphogenetic proteins (BMP-2, BMP-4, and BMP-7), and sonic hedgehog (Shh) protein.^{26,27,48} Fibroblast growth factor 4 is a potent stimulator of epithelial and mesenchymal cell proliferation.²⁵ Epithelial and ectomesenchymal cells adjacent to the EK continue to divide in response to FGF-4, while the EK cells, which produce FGF-4, remain nondividing. The cells of the EK are retained in the G1 phase of the cell cycle by a high level of expression of the cyclin-dependent kinase inhibitor, *p21*. Bone morphogenetic protein 4 may regulate EK activity via its ability to sustain high levels of *p21* expression.⁴⁷

By secreting growth factors, the EK promotes cell proliferation along a proximodistal axis, leading to

the formation of a cusp. In this sense, the EK is akin to the apical ectodermal ridge that controls limb bud development. In establishing coronal form, embryonic dental tissues follow a pattern of polarized growth. Cells in the cervical loop proliferate and move away from older differentiating cells located nearer to the cusp tip.

The best example of polarized growth is found in the developing limb. The specific genes that participate in determining the anteroposterior axis of developing limbs are also expressed in cap to bell stage tooth buds. The *Shh* gene responsible for polarizing activity in developing limbs is active in the enamel knot (see Fig 1-12) and in differentiating odontoblasts and ameloblasts.⁴⁸ Proof that genes that regulate polarized growth, such as *Shh*, are active in the tooth bud was obtained when tooth buds were grafted to developing limbs. The grafted tooth buds induced the formation of additional digits, revealing a capacity for polarizing growth in an anteroposterior axis.⁴⁸

In multicusped teeth, secondary EKs are formed over the tips of the future cusps (see Fig 1-12). In mouse molar teeth, the EKs remain active for about 24 hours before undergoing apoptosis.⁴⁹ Programmed cell death is also responsible for the removal of the dental lamina after tooth bud formation.

Figs 1-13a and 1-13b Role of vitamin A during tooth formation.

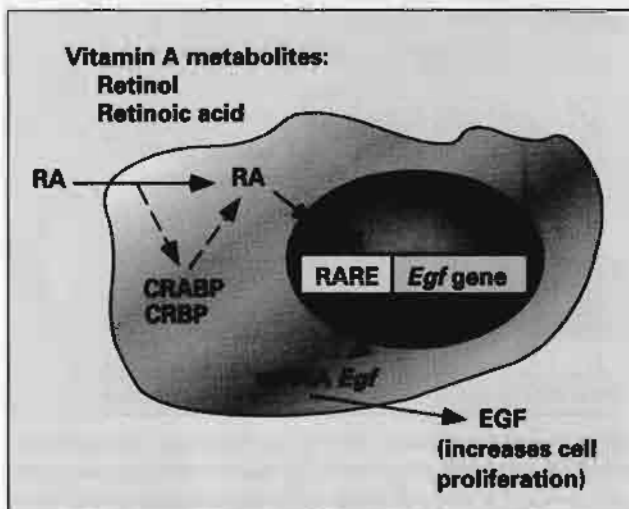


Fig 1-13a Cellular action. Retinoic acid (RA), the major active metabolite of vitamin A, diffuses into the cell interior, where it binds to cellular retinoic acid-binding protein (CRABP), or, if the level of CRABP is low, may enter the nucleus to interact with its receptor (RAR). Retinoic acid receptors activate retinoic acid response elements (RARE) that regulate gene transcription, thereby stimulating the production of messenger ribonucleic acid (mRNA). The epidermal growth factor gene (*Egf*) is regulated by a RAR-RARE complex. The increase in cell proliferation effected by vitamin A is believed to be the result of the secretion of epidermal growth factor (EGF), a known mitogen for dental epithelium and ectomesenchyme. (CRBP) Cellular retinol-binding protein.

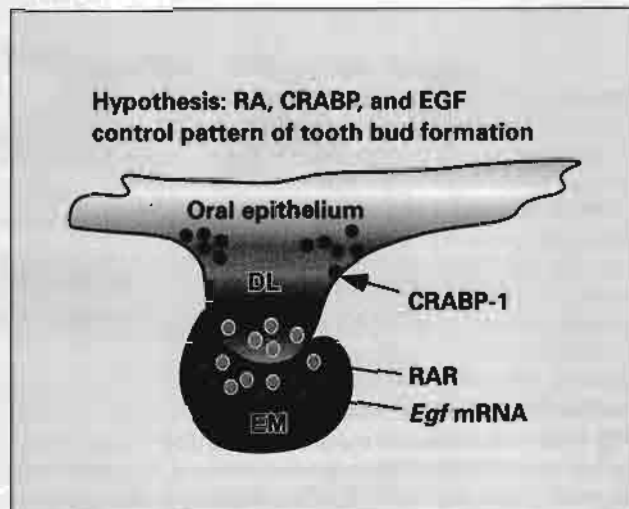


Fig 1-13b Tissue expression. Proposed model by which vitamin A can set the location of the dental lamina (DL). Cellular retinoic acid-binding proteins (CRABPs) expressed in epithelium adjacent to the DL limit the availability of retinoic acid (RA) for interaction with retinoic acid receptors (RARs), while the level of expression of CRABPs is low in the DL, permitting RA stimulation of epidermal growth factor (*Egf*) gene transcription in the DL and the adjacent ectomesenchyme (EM). (mRNA) Messenger ribonucleic acid; (EGF) epidermal growth factor.

Growth and Differentiation Factors That Regulate Tooth Formation

Bone morphogenetic factors, Shh, and FGFs are also important during the later stages of tooth development.⁴⁷ Both BMP-2 and BMP-7 are expressed in the IEE across from the differentiating odontoblasts, suggesting that they may have an inductive role. Secretory odontoblasts express BMP-4 and BMP-7, while BMP-5 appears to be restricted to fully differentiated ameloblasts. Bone morphogenetic protein 3 is localized in the cells of the dental follicle.

Activin A, a protein structurally related to BMPs and a member of the TGF- β superfamily of cytokines, has been implicated in signaling during tooth development.⁵⁰ Mice deficient in activin A have craniofacial abnormalities and failure of incisor tooth development.

Vitamin A and its metabolic derivatives, retinol and retinoic acid (RA), are essential regulators of epithelial cell proliferation and differentiation and have special impact on tooth development.⁵¹⁻⁵⁴ The importance of vitamin A in the initiation of tooth development was underscored by the observation that when endogenous vitamin A is blocked in vitro, the dental lamina fails to develop in organ cultures of mouse embryonic mandibles.⁵⁵ Early studies of the effect of vitamin A on tooth development showed that a deficiency of the metabolite leads to defective enamel and dentin.⁵⁶ In contrast, excessive vitamin A increases the chance for tooth bud fusion and/or the formation of supernumerary teeth.^{57,58}

In organ cultures of embryonic mandibular explants, retinol and retinoic acid increase epithelial proliferation and stimulate the formation of extra tooth buds. Retinoic acid exerts its effect by binding to nuclear transcription factors (RA receptors

[RARs]) located near retinoid response elements on various target genes, one being the gene that produces epidermal growth factor (EGF) (Figs 1-13a and 1-13b).⁵⁹ Retinoic acid also increases the expression of midkine (MK) protein, a regulator of cell proliferation.

Cellular retinol-binding proteins (CRBPs) and cellular retinoic acid-binding proteins (CRABPs) are involved in the metabolism and storage of vitamin A metabolites in the cytoplasm. Cellular retinol-binding proteins and CRABPs may control the level of free RA available to interact with the nuclear RARs. Because a nuclear RAR and an RA response element control the gene responsible for coding EGF, the ability of RA to increase cell proliferation may be mediated through increased EGF production (see Figs 1-13a and 1-13b). The site-specific increase in epithelial cell division required for the formation of the dental lamina and the subsequent development of tooth buds could be controlled by localized production of EGF in response to RA.^{51,53,54,60}

Both RARs and CRABPs have been localized in the dental lamina and adjacent ectomesenchyme as well as in dental epithelium and ectomesenchymal components of developing teeth (see Figs 1-13a and 1-13b).^{51,53,54} In addition, CRABPs have been localized in the epithelium adjacent to sites of dental lamina formation, suggesting that RA may be bound at such sites. In the dental lamina, where there appears to be fewer CRABPs, the RA molecules are free to interact with their nuclear receptors and thereby increase the expression of EGF.⁵⁴

Epidermal growth factor, acting in a paracrine or autocrine manner, appears to control the rate of cell proliferation in the early stages of tooth development. Epithelial cells of the dental lamina and early enamel organ express EGF receptor.⁶¹ When the enamel organ reaches the cap stage of development, the level of binding of EGF decreases in the epithelial cells but increases in the ectomesenchymal cells of the underlying dental papilla. The importance of EGF in tooth development is underscored by the observation that interfering with the synthesis of EGF blocks odontogenesis.⁶²

Another RA-regulated gene expressed during tooth development is midkine (MK).^{63,64} This gene codes MK protein, a heparin-binding growth and differentiation factor unrelated to two other heparin-binding molecules, fibroblast growth factor, and hepatocyte growth factor. The MK gene and its product are preferentially located in embryonic tissues undergoing epithelial-mesenchymal interaction. Both MK mRNA and MK protein are preferentially expressed in

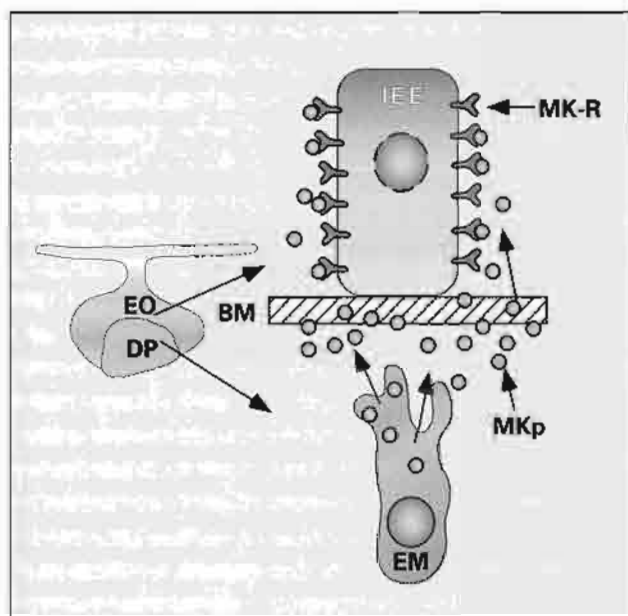


Fig 1-14 Appositional pattern of the expression of the midkine (MK) gene in the ectomesenchyme (EM) and the localization of the MK protein (MKp) to the surface of the inner enamel epithelial cells adjacent to the basement membrane (BM) of a cap stage tooth bud. The diffusible MK protein is concentrated in the BM and is bound to cell surface receptors (MK-R) on epithelial cells, where it may act as a paracrine-signaling molecule. Although EM cells make MK protein, they appear to lack receptors. (IEE) Inner enamel epithelium; (EO) enamel organ; (DP) dental papilla. (Adapted from Mitsiadis et al⁶³ with permission from The Company of Biologists.)

all stages of developing maxillary and mandibular teeth of embryonic mice. The differential or appositional localization of MK mRNA and MK protein in developing dental ectomesenchyme and its receptor on the cells of the IEE provides an instructive example of epithelial-mesenchymal interaction (Fig 1-14). During the cap stage of tooth development, the MK protein is secreted by the ectomesenchymal cells and concentrated in the basal lamina. The MK protein binds to MK receptor, acting as a paracrine regulator of cellular activity in the IEE (see Fig 1-14).

Midkine appears to regulate cell proliferation, possibly by inhibiting cell division in preparation for cell differentiation. The significance of MK in tooth development is confirmed by the observation that antibodies to MK inhibit odontogenesis.^{63,64} The highest levels of MK are observed in the IEE, its basal lamina, the dental papilla, and especially in differentiating odontoblasts. With the onset of dentin secretion, MK is no longer detectable in odontoblasts or in the differentiating preameloblasts.

Evidence continues to accumulate that reciprocal interaction via diffusible signaling molecules, as exemplified by MK, regulates epithelial-mesenchymal differentiation. A similar pattern of expression and localization has been reported for TGF- β , hepatocyte growth factor, and BMP during tooth development.

Neurotrophins and neurotrophin receptors are expressed in developing teeth in association with differentiating preameloblasts and preodontoblasts.^{65,66} They are also expressed in the subodontoblastic layer. Neurotrophins play a central role in the development and maintenance of nerves. Recent studies suggest that neurotrophins are expressed in early dental epithelium before the developing teeth are innervated.⁶⁷ The presence of neurotrophins and their receptors in developing teeth, and their changing spatiotemporal distribution, suggest that, in addition to a role in dental neuronal development, they may have other non-neuronal regulatory functions. Evidence obtained in other developing organ systems has indicated that neurotrophin receptors also bind matrix molecules and could act in an adhesive capacity during cell migration and/or condensation.

Nerve growth factor is a ligand for the tyrosine kinase receptor A member of the neurotrophin receptor family. Nerve growth factor produced in the developing tooth may act locally to control the number of cell cycles in the IEE and dental papilla proliferation compartments. The expression of nerve growth factor receptor decreases as cell division in the IEE ceases prior to ameloblast differentiation.⁶⁸

Growth hormone, growth hormone-binding protein, and growth hormone receptor have been localized in developing teeth. Cells of the enamel organ and dental papilla appear to be targets for growth hormone. Increased staining for growth hormone and its receptor was observed in differentiating cells of the IEE and the preodontoblastic layer of the dental papilla.⁶⁹ Likewise, insulin-like growth factor is concentrated in the IEE and dental papilla during ameloblast and odontoblast differentiation.⁷⁰ Hepatocyte growth factor and its receptor are expressed in the dental papilla.⁷¹ Hepatocyte growth factor acts as a mitogen in regulating cell proliferation in the enamel organ and dental papilla. Antisense nucleotides to hepatocyte growth factor reduce mitotic activity in the IEE and dental papilla, leading to abnormal tooth development.

The neurotransmitter serotonin (5-hydroxytryptamine) is another potential morphogenetic signaling molecule. Specific uptake of serotonin occurs transiently in oral epithelium and developing teeth.⁷²

Tooth buds grown in the presence of inhibitors of serotonin uptake fail to develop beyond the bud stage.

Continued research of the signaling events initiated by growth factors and matrix molecules will soon lead to a more complete understanding of tooth development. According to Slavkin,⁷³ "Recent advances towards identifying epigenetic signals such as growth factors, regulatory or homeotic genes, and the significant advances towards understanding how cis- and trans-regulating elements control differential gene expression during development provide enormous optimism for future research in craniofacial genetics and developmental biology."

Establishing Coronal Form (Cusp Formation)

As noted earlier, the three-dimensional plane of the IEE basal lamina sets the position of the dentino-enamel junction and thus the anatomic shape of the crown. From the cap stage, the enamel organ continues to increase in size until it assumes a bell-shaped structure, almost completely enclosing the dental papilla (see Fig 1-1). The three-dimensional shape of the enamel organ, at various stages of its development, has been precisely reconstructed from serial sections of human embryos. In extensive studies of human embryos, Ooe⁷⁴ has demonstrated that secretion and mineralization of dentin and enamel matrices begin only after the shape of the crown has been determined in soft tissues.

Numerous factors under genetic control, including rates of cell division, assembly of cytoplasmic contractile filaments in differentiating preameloblasts, and the osmotic pressure of the surrounding tissues, act to shape the three-dimensional topography of the basement membrane between the IEE and the dental papilla. Cusp outline is set by the three-dimensional folding of the IEE basement membrane, setting the position of the future dentino-enamel junction. Cells in both the preameloblast and preodontoblast compartments must stop dividing to differentiate into matrix-producing ameloblasts (enamel) and odontoblasts (dentin) (Fig 1-15). Proliferation is controlled from primary and secondary enamel knots established over the tips of the future cusps. The FGF-4 and EGF produced by the nondividing cells of the EK may diffuse laterally to regulate cell proliferation in the IEE and the underlying preodontoblasts (see Fig 1-12).

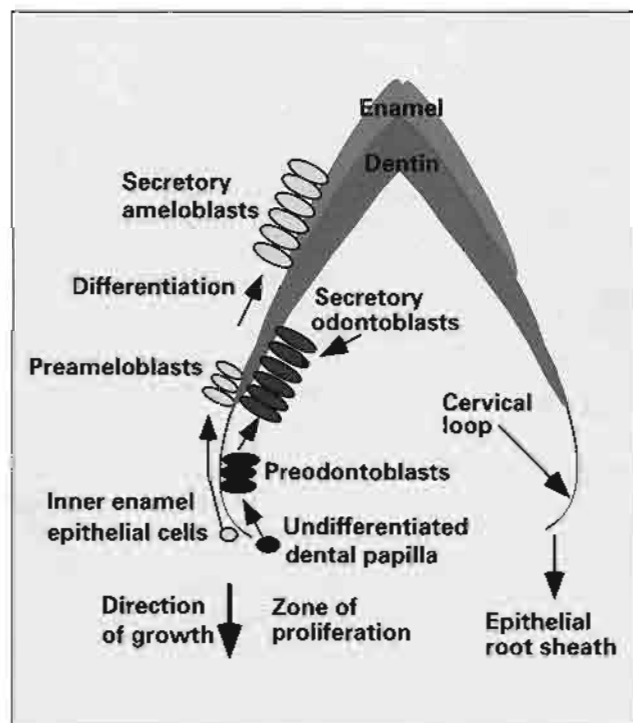


Fig 1-15 Proliferation of preodontoblasts and preameloblasts from undifferentiated precursors in the dental papilla and inner enamel epithelium located in the cervical loop area. Cell cohorts leave the proliferation compartment and differentiate into mature secretory cells. Odontoblast differentiation and dentin deposition occur slightly in advance of ameloblast differentiation and enamel matrix secretion.

Apoptosis of epithelial cells in the EK terminates cusp growth.⁴⁸ As the enamel knot begins its apoptotic decline, its function is transferred to the stratum intermedium. Progressing away from the tip of the cusp, in the proximodistal direction, a wave of signaling activity occurs in the cells of the stratum intermedium that promotes the cell proliferation necessary to complete the morphodifferentiation of the bell-shaped crown.

Cell division at the cervical loop extends the size of the enamel organ until it reaches its mature state as a bell-shaped organ almost encompassing the dental papilla. Harada et al⁷⁵ have demonstrated the presence of stem cells in the stellate reticulum of the cervical loop. Each division of a stem cell creates two daughter cells; one remains within the stem cell pool while the other cell enters the transit-amplifying pool (preameloblasts) within the IEE. A signaling pathway involving Notch and its ligand (Lunatic fringe) plays a central role in determining daughter cell entry into the

differentiation pathway.^{75,76} Odontoblasts differentiate slightly in advance of ameloblasts, forming a thin layer of predentin prior to the start of enamel secretion.

Basic Science Correlations

Cell migration

Embryonic development involves orderly and precisely timed cell migrations. In many cases, cells must move over long distances. Some migrations contain large cohorts of cells moving over relatively long distances, as in the migration of neural crest cells from specific sites in the neural tube of the head region to their final destination in the developing face and jaws. Another example is the migration of pigment cells from the neural crest to sites throughout the epidermis. Tooth development requires the migration of neural crest ectomesenchyme to appropriate locations in the developing jaw. During root development, cells of the dental sac migrate toward the newly deposited dentin surface prior to cementogenesis.

For decades, developmental biologists sought answers to the following questions: What is the basis of cell motility? What guides a migrating cell to its ultimate destination? Although the answers to these questions are still incomplete, rapid progress is being made in understanding the molecular basis of cell migration. Directed cell locomotion is a complex process. It requires plasma membrane cycling or flow, the interaction of cell surface integrins with components of the extracellular matrix as well as the cytoskeleton, and the contraction of actin and myosin filaments.^{77,78} It also requires receptor-ligand signaling systems to detect and respond to gradients of chemotactic molecules.

Some cell types are relatively stationary, while other types engage in locomotion (neutrophils and lymphocytes).⁷⁹ Transmigration through the extracellular matrix is a result of the cell's capacity to explore its immediate environment. It does this through the extension of probing cytoplasmic processes (lamellae and filopodia).⁸⁰ Lamellae are flat folds of cytoplasm sent out across a broad area, while filopodia are narrow fingerlike protrusions (Fig 1-16).

The extension and retraction of lamellae and filopodia are, in part, responses to two fundamental properties of the cell: the continuous turnover of the plasma membrane, and the contractility of cytoplasmic microfilaments. When cell processes from a region of the cell boundary make adhesive contact

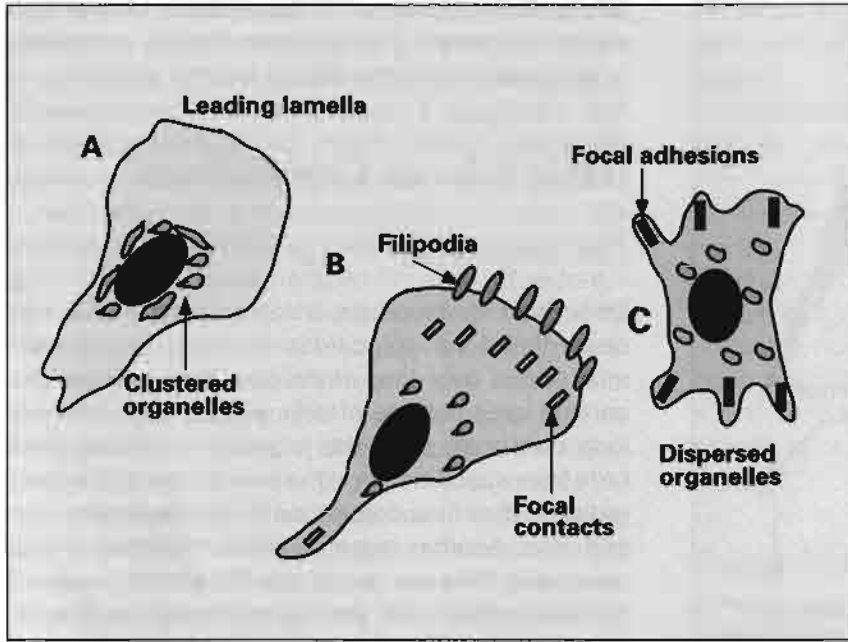


Fig 1-16 Changes in shape and cell-to-substrate contacts made by chick heart fibroblasts explanted onto plastic culture dishes. (A) In the early phase of migration, the cells exhibit a clear leading lamella devoid of dense focal contacts. Only close contacts are made at this stage. (B) With time, the cells establish filipodia and focal contacts at the leading edge. A tail of trailing cytoplasm is characteristically found on migrating fibroblasts. (C) After 3 days in culture, most cells no longer have the migratory phenotype, no leading lamella is observed, and many well-developed focal adhesions are present in many regions of the cells. (Adapted from Couchman and Rees⁶¹ with permission from The Company of Biologists.)

with a substrate, cytoplasmic polarity is established toward the substrate, and new membrane is transported toward that surface. This region of the cell surface has the potential of becoming the leading edge if there is no impediment to prevent the cell from moving forward in that direction. New membrane is added to the leading edge of the cell and retrieved toward the center of the cell.

It has been calculated that the lipid phase of the plasma membrane of a fibroblast turns over in about 50 minutes. Some intramembrane proteins are caught up in this flow, while others remain in place because of their association with the internal cytoskeleton or with extracellular substrates.

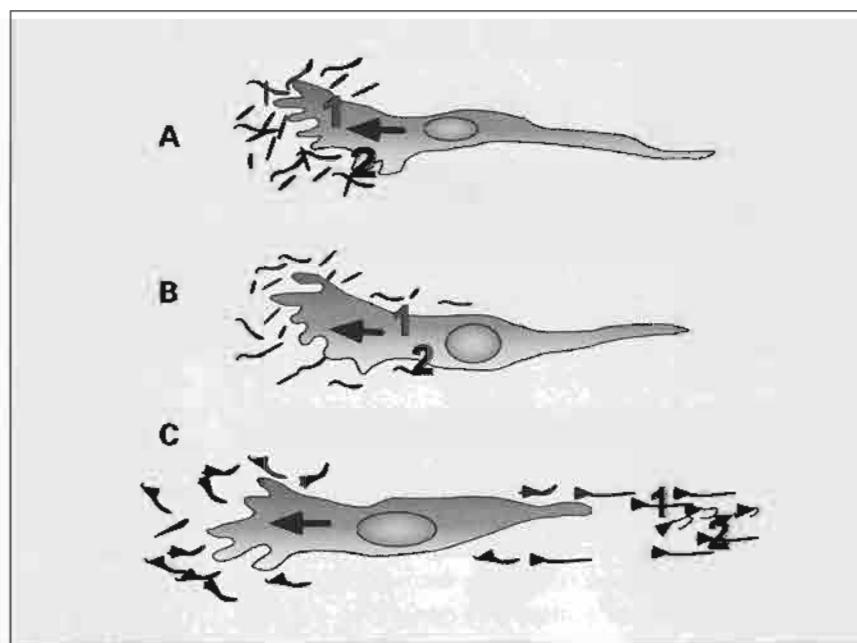
Protrusion of lamellae and filipodia at the leading edge is driven by rapid polymerization of actin filaments (see chapter 11 for a discussion of actin filament formation). Assembly of linear actin bundles may push the membrane outward or cause an increase in local hydrostatic pressure to deform the membrane outward at the leading edge. Because calcium triggers actin polymerization, it has been proposed that filipodial formation at the leading edge might be regulated by the entry of calcium ions through cell membrane channels.

Another explanation for the forward extension of the plasma membrane is the assembly of new membrane via exocytosis at the leading edge and the simultaneous endocytosis toward the middle and rear of a migrating cell. Polarized exocytosis-endocytosis cycles have been observed in migrating fibroblasts and neurite growth cones.

To develop traction and forward movement, cells must form attachments between their leading edge and the substratum. Cells migrating *in vitro* on glass cover slips make close contacts and focal contacts with the surface of the glass.⁸² At close contacts, the cell membrane is separated from the substratum by a space of 20 to 30 nm. Close contacts represent the initial association of specific cell membrane attachment proteins to the extracellular matrix. Close contacts are typically found at the very leading edge of lamellae and filipodia.

In contrast, focal contacts typically occur just distal to the outer zone of the leading edge (Figs 1-16 and 1-17). In focal contacts, the cell membrane is only 10 to 15 nm from the surface of the substrate. The focal contact is the product of the maturation of the close contact by recruitment of integrin receptors and other membrane-associated proteins. Along with

Fig 1-17 Hypothesis proposed by Harris (1973) to explain how the forward movement of cells is coordinated to the development of stable cell-to-matrix contacts associated with actin and myosin filament bundles. (A) Focal contacts (1 and 2) established at the leading edge remain in position as (B) new membrane and cytosol advance in the continued protrusion of the leading lamella. (C) With time, the focal contacts, first established at A, become located at the trailing end of the cell, and will eventually be ruptured as the tail is pulled forward. The detached focal contacts with bits of cytoplasm remain attached to the substratum. Contraction of actin and myosin in the cell body propels cytosol forward to the leading lamella. In the process, matrix molecules become aligned parallel to the direction of cell migration. (Adapted from Hay.⁸⁸)



the integrins, actin, vinculin, and talin are rapidly associated with the initial site of attachment to form a focal contact or focal adhesion. Thus, the integrins mediate transmembrane linkage of the cytoskeletal proteins to the extracellular matrix.⁸³

The integrin dimer $\alpha 5 \beta 1$ represents one type of integrin fibronectin receptor. Fibronectin participates as the extracellular component of the close contact in migrating fibroblasts and neural crest cells.⁸⁴ Motile cells make cell-to-matrix attachment interactions of a transient nature (close contacts). Fibronectin receptors tend to be more dispersed over the surface of migrating cells.

Cell-to-cell attachments and stable cell-to-matrix adhesions (focal adhesions) assume greater importance in stabilizing nonmotile cells at their final destination. In stationary cells, the fibronectin receptors are clustered in alignment with extracellular fibronectin fibrils.^{85,86} When cells are attached to matrix fibrils, which are under tension, the cells develop large focal adhesions (fibronexus) associated with cytoplasmic actin and myosin bundles (stress fibers). The fibronexus junction is described in chapter 6.

Specific extracellular matrix molecules, organized into three-dimensional scaffolds, provide pathways

for the selective migration of certain cell types. Neural crest cells migrate in defined tracks rich in fibronectin and hyaluronic acid. The same is true for the migration of fibroblasts into the primary corneal stroma. The basal lamina, or substances associated with it, can also act as a substrate for the preferential migration of cells in vivo. Certain types of neural crest cells end their migration when they encounter regions rich in tenascin, a large extracellular attachment molecule.

Several environmental stimuli cause cells to undergo directed migration. Cells can move along a concentration gradient of an extracellular matrix molecule (haptotaxis). In an electrical field, cells migrate toward the cathode (galvanotaxis). Fibronectin fragments induce directed migration of fibroblasts, a stimulus likely to be important in wound healing.⁸⁷

Cells also tend to move outward from a cell mass. Cells on the perimeter of the cell mass continue to form leading lamellae and filopodia along their free surface and thus are able to move away from the cell mass. Within the cell mass, however, cells are contact inhibited; a state of reduced membrane ruffling and filopodial extension occurs along the adjacent

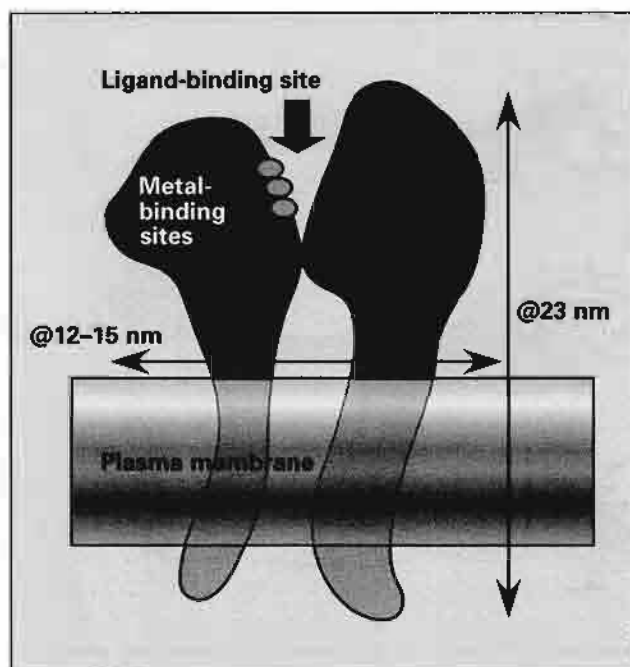


Fig 1-18 Integrin-type receptors. The α and β integrin transmembrane proteins form a dimer with a shared ligand-binding site. Metal-binding sites on the α subunit are needed for receptor function.

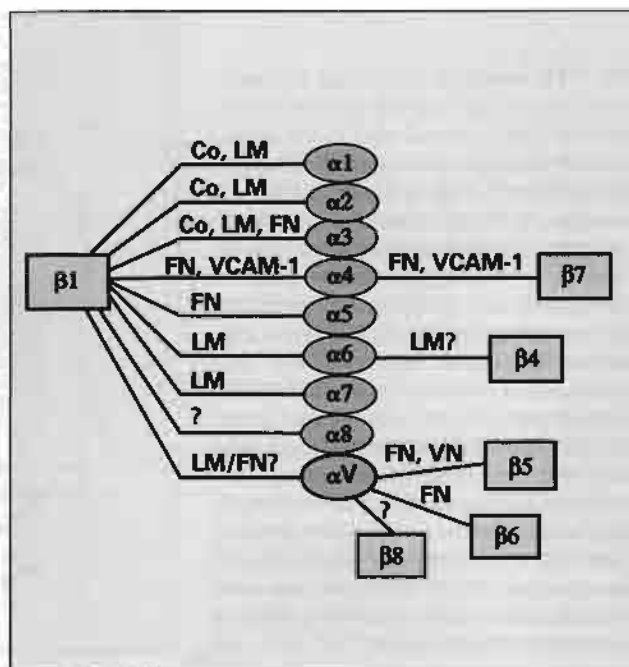


Fig 1-19 Integrin molecules of the very late activation subfamily. Heterodimers of β and α subunits form cell surface receptors interacting with various extracellular matrix adhesion molecules. (Co) Collagen; (FN) fibronectin; (LM) laminin; (VCAM-1) vascular cell adhesion molecule 1; (VN) vitronectin. (Adapted from Amaout⁹¹ with permission from Elsevier Science.)

surfaces of juxtaposed cells. Directed migrations of neural crest cells within the extracellular matrix scaffold proceed from areas of high to low cell density because of contact inhibition.

Extracellular matrix molecules may undergo reorganization following interaction with the cell surface of a migrating cell (see Fig 1-17).^{84,88-90} Traction transmitted to the extracellular matrix by migrating (contracting) cells also exerts an organizational influence over matrix molecules. As fibroblasts migrate through a collagen gel *in vitro*, they cause the extracellular collagen fibrils to become aligned parallel to the long axis of the fibroblasts and the gel to contract. Fibronectin fibrils increase in size and organization toward the trailing edge of migrating fibroblasts. The role of cell polarity and migration in determining the organization of collagen in the periodontal ligament is discussed in chapter 6.

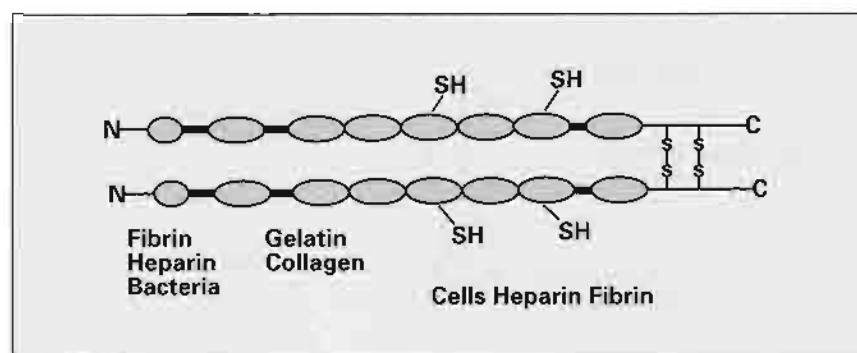
Cell and substrate adhesion molecules

Calcium-dependent cadherins, integrins, selectins, plasma membrane proteoglycans, and members of the immunoglobulin superfamily, such as neural cell adhesion molecule, participate in forming cell-to-cell and cell-to-matrix adhesions.⁹¹ Members of these transmembrane proteins play essential roles in the cellular organization of tissues and organs and in the migration of cells in embryonic and adult tissues.⁹¹⁻⁹³ The cadherins, components of desmosomes, are discussed in chapter 4, and the selectins, adhesion molecules that regulate leukocyte emigration from blood vessels, are described in chapters 13 and 14.

Integrins

The integrins are a family of cell surface transmembrane proteins that developed very early in evolution⁹¹⁻⁹⁴ (Figs 1-18 and 1-19). Integrins are heterodimers made up of α and β subunits. At least 14

Fig 1-20 The elongated fibronectin molecule is made up of two similar subunits. Each consists of globular domains joined by flexible polypeptide sections. Specific binding sites have been mapped on the molecule for various cells and molecules as shown.



α and eight β subunits have been identified. Figure 1-19 contains a chart of the subunits and ligands of the very late activation-type integrins.

Both integrin subunits are transmembrane proteins. The extracellular globular domains are larger than the cytoplasmic and intramembrane segments (see Fig 1-18). The extracellular portion of the α subunit contains metal-binding sites necessary for receptor function. The combined external globular domains of the α and β subunits form the ligand-binding site. Some integrins bind more than one type of ligand; for example, the $\alpha 1 \beta 1$ integrin binds to both collagen and laminin (see Fig 1-19). It is also apparent that individual ligands, such as fibronectin, are recognized by several integrins.

Cells use integrins to adhere to a variety of extracellular matrix molecules and to communicate chemically in a bidirectional way with their environment. Information from the extracellular matrix is gathered when ligands bind to the extracellular portion of the integrins, producing conformational changes in the cytoplasmic portion of the molecules and thereby altering their interaction with adjacent cytoplasmic molecules. Ligand binding to integrins can also exert an intracellular effect through the activation of tyrosine kinases.

Conversely, the binding of certain cytoplasmic proteins to the cytoplasmic domain can induce conformational changes in the external part of the integrin molecules, affecting their affinity for extracellular ligands. Through this process, the cell can interact with its environment, creating adhesive contacts and/or activating specific differentiation cascades.

The expression of integrin receptors for laminin has been shown to oscillate between IEE and dental papilla ectomesenchyme during tooth formation.⁹⁵ Whether integrin-laminin signaling pathways have a significant role in ameloblast differentiation remains

to be determined. Additional discussions of the role of integrins in cell activation and muscle differentiation are contained in chapters 11, 13, and 14.

Syndecan

Syndecans are integral membrane proteoglycans. Four types have been identified based on differences in the core protein. Each syndecan molecule consists of a short cytoplasmic domain, a helical hydrophobic domain inserted into the plasma membrane, and a large extracellular domain containing several glycosaminoglycan side chains.

Syndecan 1 is typically located in epithelia and in embryonic mesenchymal tissues, especially in areas of epithelial-mesenchymal interaction, such as in developing teeth.⁹⁶ Because of its binding interaction with tenascin, it may play a role in condensation of ectomesenchymal cells to form the dental papilla.³⁷ In addition to binding tenascin, syndecan 1 also binds fibronectin, and collagen types I, III, and V.

Syndecan 4 is the smallest and most widely distributed type of syndecan. It colocalizes with integrins in focal adhesions to extracellular fibronectin. Syndecans are not only matrix receptors but also coreceptors for growth factors and cytokines, capable of potentiating signal transduction events.

Fibronectin

Fibronectin is a large extracellular glycoprotein with multiple binding sites capable of forming attachments to cells, collagen, heparin, fibrin, tenascin, bacteria, and other proteoglycans.^{97,98} Fibronectin has a dimeric structure composed of two equal polypeptide chains joined near their C-terminal by disulfide bonds. Binding sites on each chain have been identified for cell membrane integrins and a variety of extracellular matrix molecules (Fig 1-20). Fi-

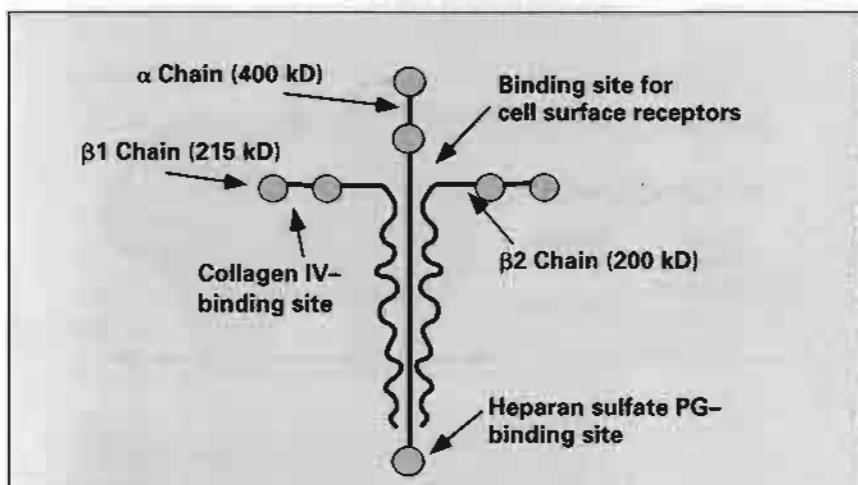


Fig 1-21 Structure of the laminin molecule.

bronectin is a significant component of basement membranes in developing organ systems, where it stabilizes cells and thereby permits them to establish polarity and to undergo further differentiation. A good example of this type of interaction occurs during the differentiation of the preodontoblasts.

The interaction of cells with fibronectin is important not only during embryonic development but also in the migration and stabilization of cells in the adult organism. Fibronectin plays an important role in wound healing by interacting with fibrin to create a scaffold for the migration of fibroblasts. Fibronectin stimulates fibroblast invasion of collagen gels. The gelatin-binding domain of the fibronectin molecule is essential to this migratory action. The gelatin-binding domain segment interacts with a fibroblast surface integrin protein to induce a transition to the migratory phenotype.

The recognition site of the cell-binding domain of fibronectin has been identified to consist of the tripeptide, arginine-glycine-aspartic acid (the RGD sequence). This sequence binds to the cell membrane integrins (fibronectin receptors). The $\alpha 5 \beta 1$ integrin is the main fibronectin receptor. The association of integrin fibronectin receptors to extracellular fibronectin triggers the recruitment of cytoskeletal and signaling molecules to the plasma membrane site of attachment to form focal adhesions. Fibronectin is concentrated at the IEE basal lamina and along the cytoplasmic surface of preodontoblasts.^{4,99-101} The role of fibronectin and its receptor in odontoblast differentiation is discussed in chapter 2.

Laminin

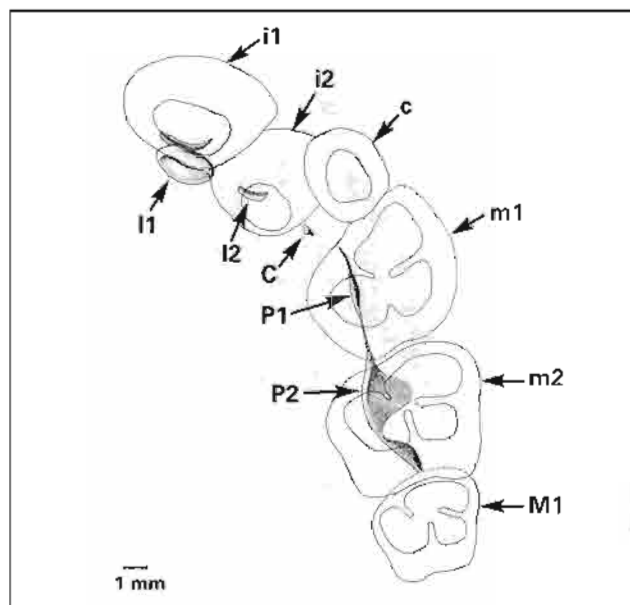
Laminin is a major constituent of the basal lamina complex. It is a large glycoprotein with a molecular weight of about 800,000 d. The laminin molecule is a heterotrimer of $\beta 1$, $\beta 2$, and α subunits. The three chains assemble to form a cross-shaped molecule (Fig 1-21).¹⁰² Laminin binds to type IV collagen, to heparan sulfate proteoglycans (perlecan) of the basal lamina, and to receptors in the cell membrane of various cells, especially epithelial cells. Laminin 5 subunits are expressed in the enamel organ, and the protein is localized in the basal lamina beneath the IEE.^{99,103}

Immunocytochemical studies reveal temporospatial changes in laminin subunit expression during odontoblast and ameloblast differentiation.¹⁰³ The results of tissue recombination experiments suggest that the dental ectomesenchyme controls the expression of laminin in the dental epithelium.¹⁰⁴ Laminin is discussed further in chapter 4.

Tenascin

Tenascin, a large extracellular matrix molecule, also known as *cytotactin* and *hexabrachion*, is made up of six polypeptide chains assembled to form a six-arm structure capable of interacting with a variety of cells and extracellular matrix molecules. Because the six polypeptide chains appear to represent separate gene products, it has been suggested that tenascin molecules may have tissue specificity.

Fig 1-22 Developing primary teeth and the primordia of the permanent teeth in a 28-week human fetus. Maxillary quadrant. (i1) Primary central incisor; (i2) primary lateral incisor; (c) primary canine; (m1) primary first molar; (m2) primary second molar; (I1) permanent central incisor; (I2) permanent lateral incisor; (C) permanent canine; (P1) permanent first premolar; (P2) permanent second premolar; (M1) permanent first molar. (Adapted from Ooe¹⁰ with permission.)



Tenascin binds to cell surface proteoglycan (syndecan). Expression of tenascin in dental ectomesenchyme coincides with the concentration of the dental papilla.^{100,105} It has been demonstrated that tenascin prevents the migration of certain neural crest cells, causing them to assume a round shape characteristic of stationary cells.

Nidogen

Nidogen (also called *entactin*) is a rod-shaped protein consisting of a single polypeptide chain, approximately 30 nm long, with globular domains at each end and one centrally located domain.^{106,107} Because nidogen binds with high affinity to collagen IV and laminin, it has an organizing role in assembly of the basal lamina. Nidogen also binds perlecan, the large heparan sulfate proteoglycan of the basal lamina.

The coexpression of laminin 1 and nidogen results in a relatively stable basal lamina. In general, laminin is produced by epithelial cells and nidogen by mesenchymal cells. Temporospatial differences in the expression of laminin and nidogen are thought to have significance in epithelial-mesenchymal tissue remodeling because of resulting changes in the stability of the basement membranes.¹⁰⁸

Basal lamina

The basal lamina is a supramolecular aggregate of type IV collagen, laminin, fibronectin, nidogen, and perlecan. They form a macromolecular network with the dual function of supporting epithelial cells and providing for a permeability barrier or filter. Meyer et al¹⁰⁹ have reviewed the role of the basal lamina in tooth development and odontoblast differentiation. The basal lamina is discussed in detail in chapter 4.

Clinical Correlation: The Human Dentition

The primary (deciduous) dentition consists of 20 teeth, five in each quadrant (Fig 1-22).^{74,110} The permanent incisors, canines, and premolars form from successional laminae that extend lingually from the primary precursors toward the midline (see Fig 1-22). The permanent molars develop from a distal extension of the dental lamina, the accessional lamina (Fig 1-23). Some dental embryologists consider the permanent molars to be members of the first dentition. Their microscopic successors undergo an aborted development.

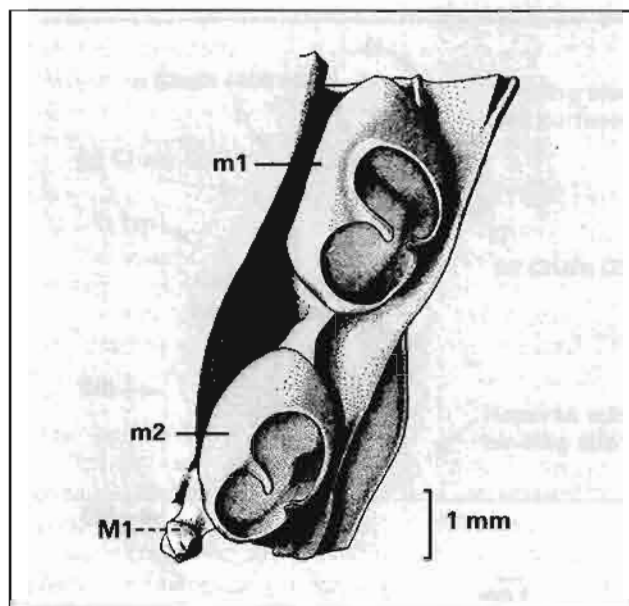


Fig 1-23 Mandibular molar region in a 159-mm fetus (at 20 weeks old), depicting the formation of the permanent first molar (M1) from a distal extension of the primordia of the primary second molar (m2). (m1) Primary first molar. (Adapted from Ooe⁷⁴ with permission.)

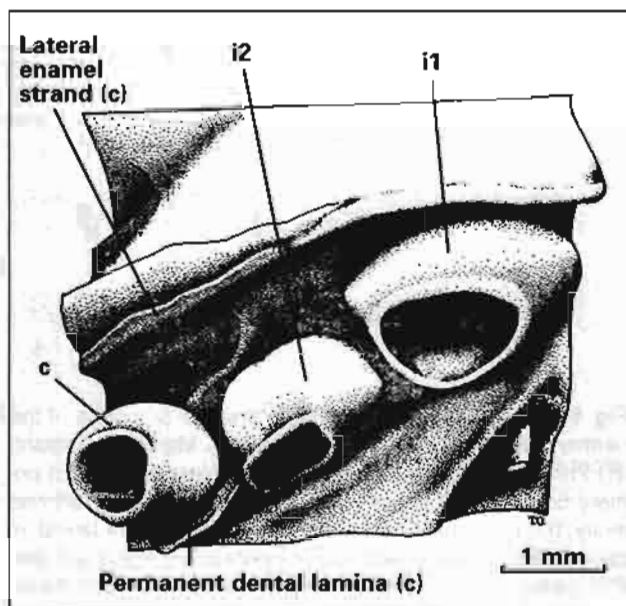


Fig 1-24 Epithelial portion of the anterior tooth germs and adjacent structures in a 144-mm fetus. (i1) Primary central incisor; (i2) primary lateral incisor; (c) primary canine. (Adapted from Ooe⁷⁴ with permission.)

During the development of primary teeth, the central incisor and canine are positioned labial to the lateral incisor (Fig 1-24). This arrangement is noted very early in the formation of the enamel organ from the dental lamina. The buds of the permanent teeth have a similar position, so that the lateral incisor is positioned lingual to the central incisor and canine. During normal postnatal development, space is created in the dental arch for the alignment of all anterior teeth. Often, the space created is insufficient, and the central incisor and the canine crowd out the lateral incisor.

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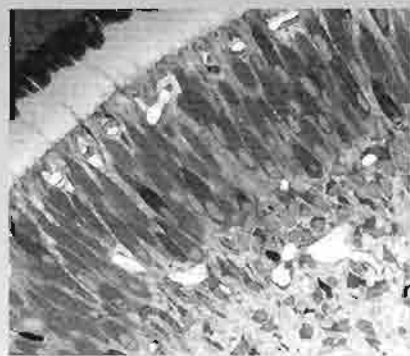
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Chapter 2

Dentin



Dentin is deposited by odontoblasts that develop ectomesenchymal cells of the dental papilla on contact with the basal lamina formed by the inner enamel epithelium.

Differentiation of Odontoblasts

Odontoblast precursors migrate into the developing jaw from the neural crest as part of a large population of ectomesenchymal cells that participate in the formation of many parts of the face and oral cavity. During the cap stage of tooth formation, the preodontoblasts concentrate adjacent to the inner enamel epithelium (IEE) of the enamel organ. Preodontoblasts exit the cell cycle and differentiate before the preameloblasts of the IEE have stopped dividing.^{1,2}

Contact with the IEE basement membrane and/or with other associated extracellular material of epithelial origin has long been held to be a requirement for initial odontoblastic differentiation.³ Recent experiments suggest that a fibronectin-rich substratum is a key requirement.⁴ Early studies implicating the importance of the basement membrane in odontoblast differentiation were reviewed by Ruch^{1,2} and Ruch et al.⁵

Aperiodic fibrils are key structures regulating the differentiation of odontoblasts. They are deposited first at the tip of the future cusp, and then apically toward the cervical border of the developing tooth. Shortly after the first aperiodic fibrils form, the preodontoblasts bind to them through leading-edge cytoplasmic processes (see Fig 1-8).^{3,6,7} As leading-edge

contacts increase in number, the preodontoblasts are immobilized across the basal lamina from the cells of the IEE. Polarity toward the basal lamina is established at this time.^{8,9} Odontoblast differentiation in organ culture fails when the basement membrane is removed by prior incubation in trypsin.³

Electron microscopy reveals that aperiodic fibrils, about 15 nm wide and 1.0 to 2.0 μm long, are attached to the basal lamina beneath the IEE. Fluorescent antibodies to collagen types I, III, IV, and VI, tenascin, proteoglycan, and fibronectin bind to basement membrane molecular components in the same location, suggesting that the aperiodic fibrils may consist of one or more of these matrix proteins.¹⁰⁻¹⁶ Similar patches of extracellular matrix have been observed adjacent to the plasma membrane of preodontoblasts.^{12,17}

Fibronectin receptors (165-kDa protein) are present in the leading-edge plasma membrane of preodontoblasts during differentiation and stabilization. Adherence of a cell surface 165-kDa fibronectin receptor appears to stabilize cytoskeletal elements, promote preodontoblast polarization, and trigger other cytoplasmic processes associated with differentiation.^{18,19} Attachment to fibronectin leads to its uptake and removal at the leading edge of the differentiating odontoblast.

Transforming growth factor β 1 (TGF- β 1), a growth factor that binds to fibronectin, is a well-known inhibitor of cell proliferation and a promoter of odontoblast differentiation and matrix synthesis. Thus, one important function of fibronectin may be to serve as a reservoir for growth factors that cause preodonto-

blasts to exit the cell cycle and to undergo differentiation. The importance of fibronectin in dentinogenesis is underscored by the observation that cells of the dental papilla can differentiate into odontoblast-like cells when grown in contact with a supporting surface that is rich in fibronectin and other soluble dentin matrix components.^{4,20}

Odontoblasts sequentially express several members of the TGF- β superfamily of growth factors and their receptors.²¹ During normal development, TGF- β 1 is expressed in the IEE before and during odontoblast polarization. Differentiated odontoblasts express receptors for TGF- β 1 and secrete TGF- β 1 into the dentin matrix.²² Loss-of-function mutations in the *Tgf- β 1* gene in mice cause dentin and pulpal pathoses.²³ Evidence accumulated over nearly 2 decades suggest that spatial and temporal interactions between cell surface receptors and extracellular matrix molecules and growth factors, such as fibronectin and TGF- β 1, provide the necessary information to coordinate odontoblast differentiation.

It has been suggested that the entry of calcium ions might act as a signal for mediating restructuring of the cytoskeleton during the establishment of odontoblast shape and polarity toward the IEE. Cell membrane ligand-gated calcium channels have been localized to the apical pole of the preodontoblasts (nearest the basement membrane).²⁴

In addition to the potential signaling effects of calcium, fibronectin, and TGF- β , there is evidence to suggest that enamel matrix proteins may serve a similar instructional role during odontoblast differentiation. The expression of enamel proteins in the IEE begins before the cells have acquired the secretory ameloblast phenotype. Electron microscopic studies have identified the presence of enamel matrix protein across the basal lamina in close contact with the apical pole of the developing odontoblasts.^{16,25} The enamel proteins, identified by antiamelogenin antibodies, are endocytosed in coated vesicles at the odontoblast cell surface.^{16,26} The potential instructive role, if any, for these enamel proteins in regulating odontoblast development is unclear.

Secretion of Dentin Matrix

Subsequent to odontoblast differentiation, the basal lamina is degraded. Application of *in situ* hybridization techniques has shown that preameloblasts and preodontoblasts express matrix metalloproteinase 2, an enzyme that degrades collagen IV and fibronectin, coincident with the removal of the basal lam-

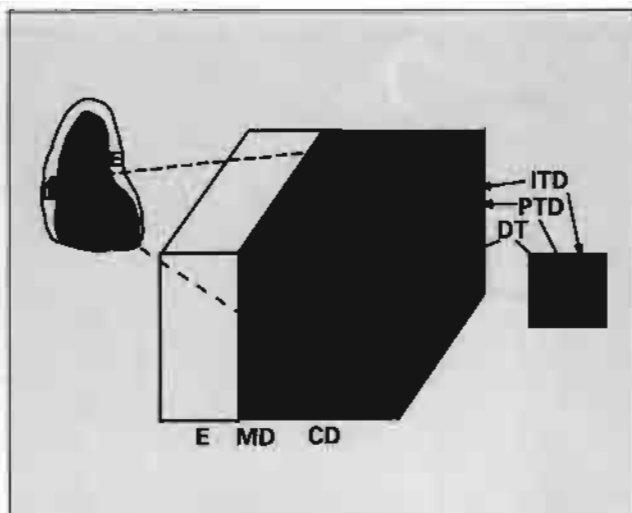


Fig 2-1 Components of dentin. The outermost layer of dentin is the mantle dentin (MD). It is deposited during the early stage of odontoblast development. With the appearance of the odontoblastic process, the major portion of dentin, the circumpulpal dentin (CD), is deposited. It consists mainly of intertubular dentin (ITD) and narrow bands of peritubular dentin (PTD) surrounding the dentinal tubule (DT). (E) Enamel; (D) dentin; (P) pulp.

ina.¹⁴ Evidence from electron microscopy suggests that the preameloblasts of the IEE phagocytose the partially degraded basal lamina.

After the breakup of the basal lamina, heterotypic cell-to-cell contacts form between cell processes of the newly differentiated odontoblasts and the distal ends of the preameloblasts. Although it was speculated that such contacts might allow the transmission of informational messages needed for differentiation, there has never been any evidence presented that functional gap junctional contacts exist between these two cell types.

In contrast, stable gap junctions and macula adherens-type junctions develop between adjacent odontoblasts during aggregation (see chapter 1).^{8,27-30} Coordination of dentin matrix secretion may require communication across gap junctions, permitting ions and small metabolites to cross from odontoblast to odontoblast. Soon after alignment of the odontoblasts, a junctional complex consisting of fascia adherens and fascia occludens forms in the distolateral cell membranes. The fascia adherens is associated with a highly developed terminal web of cytoplasmic filaments.⁸ The tight junctions of the fascia occludens do not form a zonula occludens.²⁷

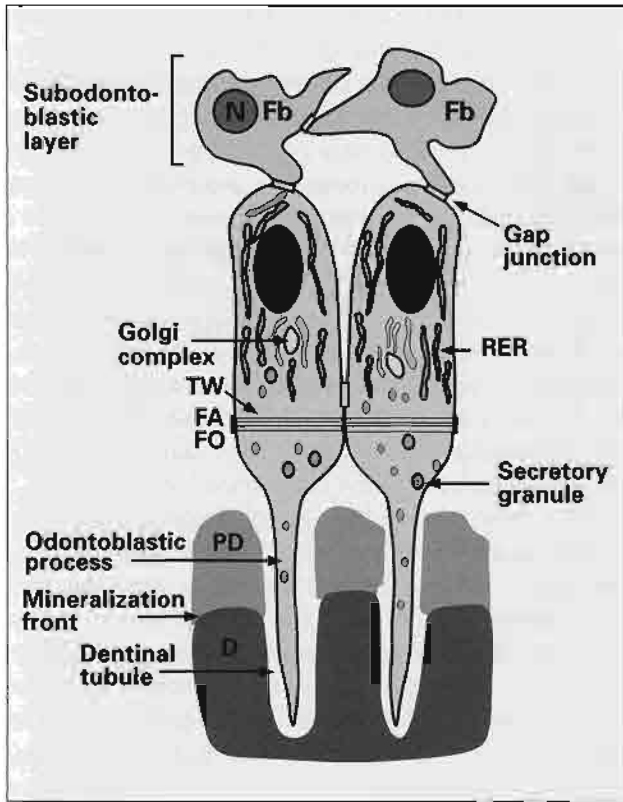


Fig 2-2 Mature secretory odontoblast. (D) Dentin; (N) nucleus; (PD) predentin matrix; (FA) fascia adherens; (FO) fascia occludens; (RER) rough endoplasmic reticulum; (TW) terminal web; (Fb) fibroblast.

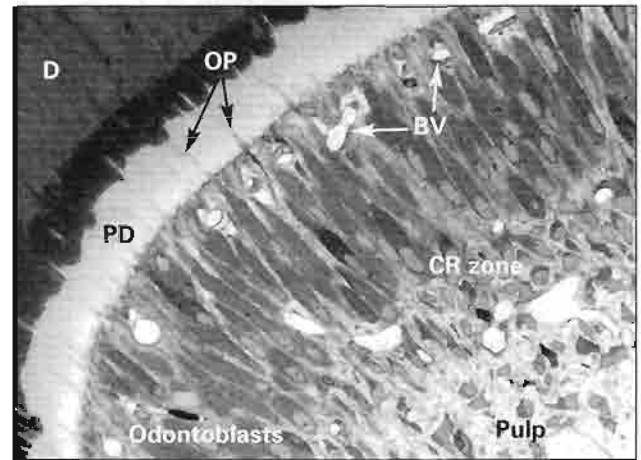


Fig 2-3 Cross section of a rat incisor, illustrating mature secretory odontoblasts. (BV) Blood vessels; (CR zone) cell-rich region of the pulp containing numerous fibroblasts; (D) dentin; (OP) odontoblastic process, (PD) predentin. (Epon section [1 μ m] stained with toluidine blue; original magnification $\times 260$.)

Concomitant with the onset of dentin matrix secretion, odontoblasts grow in length and develop large amounts of rough endoplasmic reticulum (RER). A prominent Golgi complex develops in the supranuclear cytoplasm facing the IEE. In addition to increased expression of messenger ribonucleic acid (mRNA) for collagen type I, developing odontoblasts also express mRNA for osteocalcin, dentin phosphophoryn, and high levels of alkaline phosphatase.^{31,32} As synthesis of type I collagen increases, the expression of type III collagen decreases in odontoblasts. Dentin matrix contains type I collagen and a variety of glycoproteins and glycosaminoglycans.^{33,34}

The earliest layer of dentin to form is called *mantle dentin* (Fig 2-1). The collagen fibers of the mantle dentin are thicker than those that form later in circumpulpal dentin. In coronal dentin, the collagen fibers of mantle dentin are polymerized perpendicular to the dentinoenamel junction, while the fibers of

the circumpulpal dentin form approximately parallel to the dentinoenamel junction.

As dentin matrix is deposited, the odontoblast cell body is pushed backward away from the dentin surface. A single dominant cytoplasmic process, the odontoblastic process, forms during the retreat of the cell. It remains embedded in the dentin, undergoing elongation as more dentin matrix is deposited (Fig 2-2). With the appearance of the odontoblastic process, formation of circumpulpal dentin begins.

Structure of Mature Secretory Odontoblasts

Fully differentiated odontoblasts are tall columnar cells, 50 to 60 μ m in length, characterized by a highly polarized distribution of cytoplasmic organelles (Figs 2-2 and 2-3).^{8,35,36} For descriptive purposes, it is convenient to divide the odontoblast into two parts, the

cell body and the odontoblastic process. The terminal web of cytoplasmic filaments, associated with fascia adherens junctions, provides a line of demarcation between the cell body and the odontoblastic process (see Fig 2-2). Mature odontoblasts are aligned as a single layer of columnar cells, but when crowded, as in the pulp horns or in the most incisal portion of the rodent incisor, odontoblasts assume a pseudostratified organization (see Fig 2-3).

Odontoblasts are joined and attached at their distal extremities by well-developed terminal webs and associated fascia adherens junctions (see Fig 2-2).⁸ Physical evidence of the strength of this bond is provided by the fact that the odontoblastic layer can be isolated relatively intact after demineralization and digestion of the dentin matrix. When observed macroscopically and histologically, the terminal web apparatus appears to form a continuous membranous structure. Early histologists called it the *pulpodentinal membrane*. This zone of attachment prevents the entrapment of odontoblasts in the predentin matrix and ensures that the developing surface of dentin remains relatively flat.

Although physiologic evidence suggests that a paracellular barrier to calcium exists at the distal end of the cell, no zonula occludens junction is present. Morphologic studies have revealed only a partial (fascia) occludens junction at that site. Gap junctions are formed between adjacent odontoblasts and between odontoblasts and the fibroblasts of the subodontoblast-rich zone.^{29,37,38}

The narrow intercellular spaces between adjacent odontoblasts contain collagen fibers, aperiodic microfibrils, proteoglycans, and fibronectin.^{15,39-43} These intercellular fibers (von Korff fibers) follow a spiral pathway through the interodontoblastic space, passing into the predentin between adjacent odontoblasts at interruptions of the fascia occludens and fascia adherens junctions.

During odontoblast differentiation, the RER and the Golgi complex undergo hypertrophy in preparation for protein secretion. The nucleus is restricted to the pulpal end of the cell body and is characterized by an abundant euchromatic matrix, prominent nucleoli, and many nuclear pores (see Figs 2-2 and 2-3). The RER is the major cytoplasmic organelle within active odontoblasts. Parallel cisterns of RER occupy the supranuclear cytoplasm, the borders of the Golgi complex, and the cytoplasm proximal to the terminal web (see Fig 2-2).^{8,44-46} Mitochondria are dispersed throughout the cell body.

The Golgi complex, containing aggregates of smooth-walled vesicles and cisterns, occupies a cen-

tral location (see Fig 2-2).^{5,45-47} Each stack of Golgi cisterns displays morphologic and functional polarity, with a forming face (the convex surface) and a mature face (the concave surface). The forming face develops from, and is continuously replenished by, fusion of small intermediate (transport) vesicles originating from the RER. Presecretory granules containing type I procollagen, glycoproteins, and glycosaminoglycans develop from the cisterns of the mature face of the Golgi apparatus.^{35,36,48} Phosphophoryns appear to be packaged in small, narrow vesicles.⁴⁹ The complex cytoplasmic machinery operating in the Golgi complex for targeting secretory proteins to their appropriate final destination is briefly discussed later in the chapter, in the "Basic Science Correlation" section.

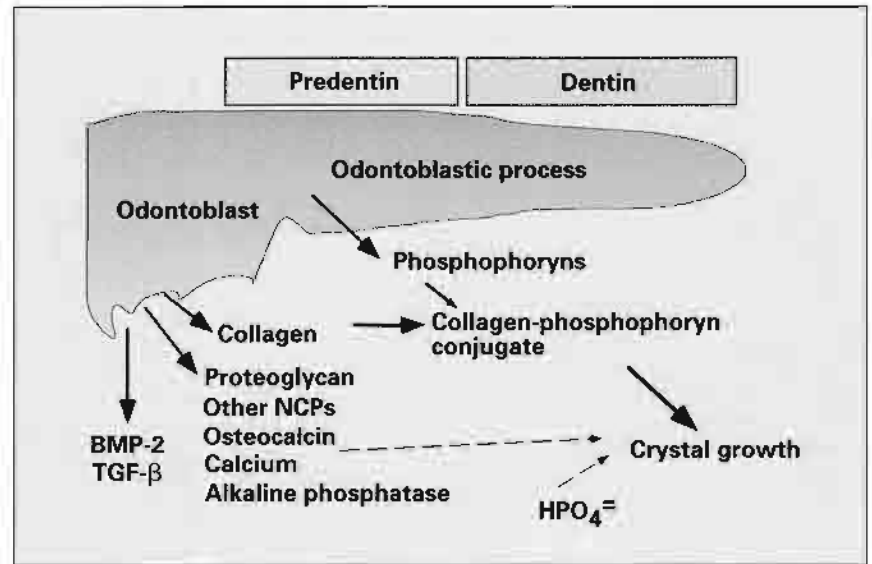
After their release from Golgi cisterns, the presecretory granules of the dentin matrix undergo condensation to form smaller secretory granules, approximately 300 nm long and 30 nm wide.^{8,35} The long axis of the secretory granule is roughly equal to the length of a type I procollagen molecule (about 280 nm long). The diameter of the granule is wide enough to contain many procollagen molecules, packaged side by side.

An essential component of the secretory machinery of the odontoblasts is its network of microtubules.⁵⁰ Interference with the assembly of microtubules prevents the migration of secretory granules from the Golgi complex to the secretory pole of the odontoblast.⁵¹⁻⁵³ The cytosolic motor-protein kinesin, using adenosine triphosphate (ATP) as an energy source, interacts with microtubules and the membranes of secretory granules to propel the secretory granules in an anterograde direction toward the secretory pole of the cell. Similar interactions between microtubules and cytoplasmic motor-proteins are involved in maintaining the organization of the Golgi complex and the polarized distribution of cytoplasmic organelles. Lysosomes and acid phosphatase are also present in mature odontoblasts, especially prominent in the distal portion of the cell body near the predentin.^{54,55}

During formation of primary dentin, the internal perimeter of the pulp becomes smaller, forcing the odontoblasts into a pseudostratified organization. With further deposition of secondary dentin, some odontoblasts undergo programmed cell death. It has been reported that half of the odontoblasts in human premolar teeth are lost over 4 years.⁵⁶

Dentin matrix is deposited in incremental amounts in a daily (circadian) biologic rhythm. These microscopic increments are visible in dentin as stripes running parallel to the mineralization front. In human dentin, the daily increment is about 4 μ m wide. Ad-

Fig 2-4 Interaction of odontoblast secretory products in predentin, dentin, and the mineralization front. Phosphate ions in phosphophoryns sequester calcium and initiate the growth of hydroxyapatite crystals. The linkage of phosphophoryns and collagen leads to deposition of mineral along the collagen fibrils. A portion of the proteoglycans are degraded and removed from the predentin before mineralization of the collagenous matrix. Growth factors (bone morphogenetic protein 2 [BMP-2] and transforming growth factor β [TGF- β]) are retained in the matrix. (NCPs) Noncollagenous proteins. (Adapted from Veis.^{22b})



ditional periodicity occurs at roughly 5-day intervals, producing the lines of Von Ebner, spaced about 20 μm apart. Circadian rhythms may contain further oscillations, which produce ultradian increments. In dentin from rodent incisors, three ultradian lines are spaced about 8 μm apart within the wider 20- μm circadian incremental lines.⁵⁷

Various explanations have been put forth to explain these rhythmic patterns of matrix deposition. Feeding and/or sleeping patterns were originally suggested to be the most likely causes of variation in secretory function. Fluctuating levels of hormones and growth factors regulated by central neural activity are the probable cause of these patterns.

Mature odontoblasts express parathyroid hormone receptors. Parathyroid hormone has an anabolic effect on odontoblasts, increasing the level of cyclic adenosine monophosphate and alkaline phosphatase.⁵⁸

Composition of the Dentin Matrix

The organic matrix of dentin contains collagen, noncollagenous proteins (proteoglycans, phosphophoryns, and glycoproteins), phospholipids, and growth factors.

Collagen

Type I collagen is the major protein of dentin matrix. Lesser amounts of types III, V, and VI collagen are also found in dentin matrix.

Electron microscopic autoradiographic studies with tritiated proline and immunocytochemical studies have shown that the procollagen of dentin matrix is secreted mainly from the predentin segment of the odontoblastic process (Figs 2-4 to 2-6).^{35,49} Tritiated proline-labeled granules accumulate in the distal part of the cell body and are discharged by a process of merocrine exocytosis. A smaller number of labeled secretory granules are present in more distal parts of the process beyond the predentin. Presumably they are secreted at sites distal to the mineralization front.

Following exocytosis of procollagen into the extracellular space, neutral proteinases remove the terminal amino and carboxy propeptides of the procollagen molecules, permitting collagen molecules to assemble into 64-nm banded fibrils of the predentin and dentin matrix (see chapter 6). Predentin matrix remains unmineralized for several days following its secretion. Typically, a layer of unmineralized predentin, approximately 10 to 20 μm thick, separates mineralized dentin from the cell body of the odontoblast (see Figs 2-2 and 2-3). A widened predentin

Figs 2-5a to 2-5d Light microscopic autoradiographs of the utilization of tritiated proline (reflecting collagen synthesis) by odontoblasts at various time periods after intravenous injection. (Original magnification $\times 500$.)



Fig 2-5a At 10 minutes, the label is at the cell periphery. (PD) Predentin; (O) odontoblast.



Fig 2-5b At 20 minutes, the Golgi complex is heavily labeled. (arrowheads) Odontoblastic process; (*) approximate location of Golgi; (D) dentin.



Fig 2-5c At 30 minutes, the grains are mostly over the odontoblastic process (arrowheads).



Fig 2-5d At 2 hours, most of the radioactivity is now in the predentin.

layer is usually a sign of abnormal mineral metabolism and/or matrix mineralization.

Fibronectin is also found in association with collagen fibrils in the predentin. Tissue inhibitor of matrix metalloproteinase 1, another secretory product of odontoblasts, is found in high concentration in predentin.⁵⁹

Noncollagenous proteins

Odontoblasts secrete noncollagenous proteins consisting of proteoglycans, phosphoporphyrins, and glycoproteins (see Fig 2-4). Electron microscopic autoradiographic localization of sulfate 35 and tritiated fucose have shown that the proteoglycan and glycosaminoglycan components of the matrix are concentrated at the mineralization front.^{29,38,48,60,61}

Biochemical and immunohistochemical studies indicate that there are specific differences in proteoglycan composition between predentin and dentin.^{62,63} Because proteoglycans interact with collagen during fibril formation, a function of predentin proteoglycans might be to regulate the size and orientation of dentin collagen fibers. It has also been suggested that predentin proteoglycans might control the timing and site of mineralization, either by sequestering calcium or by shielding potential mineral nucleation sites in the matrix. Evidence that some proteoglycans are degraded near the mineralization front by proteoglycanases and metalloproteinases supports the idea

that some proteoglycans may indeed inhibit mineralization of the dentin matrix.^{64,65}

Decorin, a chondroitin-dermatan sulfate proteoglycan with binding affinity for type I collagen, is found in dental pulp, odontoblasts, at the mineralization front, and along the mineralized walls of the dentinal tubules.⁶⁶ In contrast, decorin is conspicuously absent from predentin.

Porcine predentin matrix contains active neutral metalloproteinases (56- and 61-kDa gelatinases and 25-kDa proteoglycanase) capable of degrading proteoglycans at the mineralization front.^{64,65} The activity of these enzymes is calcium dependent. A mechanism must exist to regulate the availability of calcium for enzyme activation and matrix mineralization at the mineralization front. Endocytosis of proteoglycan degradation products, and membrane retrieval by coated vesicles, occurs in the proximal part of the odontoblastic process.

Not all of the matrix proteoglycans are degraded prior to mineralization. Chondroitin-6-sulfate, chondroitin-4-sulfate, and hyaluronate, associated with core proteins, have been extracted from demineralized dentin. Five dentin proteoglycans, ranging in size from 100 to 400 kDa and rich in chondroitin-4-sulfate, have been partially characterized by Steinfort et al.⁶³

Three noncollagenous proteins, dentin phosphoporphyrin (DPP), dentin matrix protein 1 (DMP1), and dentin sialoprotein (DSP), all contained in dentin matrix, appear to be specifically associated with biominer-

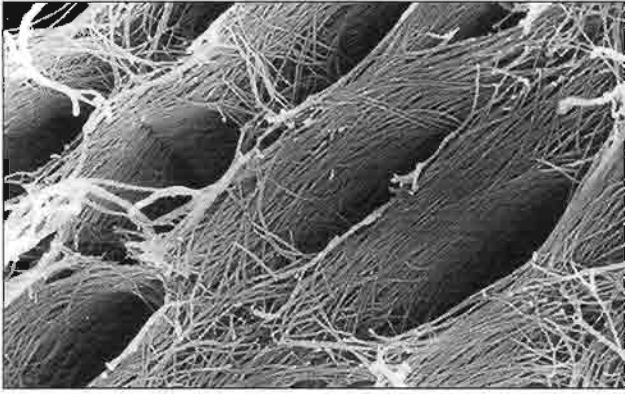


Fig 2-6a Scanning electron microscopic view of the surface of predentin after the pulp and odontoblasts have been stripped away. The oval depressions represent the spaces (dentinal tubules) previously occupied by the odontoblastic processes. Note the uniform diameter (about 3 μm) of the collagen fibrils and their orientation around and perpendicular to the long axis of the dentinal tubule. (Adapted from Tabata et al⁴³ with permission from John Wiley & Sons. Original magnification $\times 18,000$.)

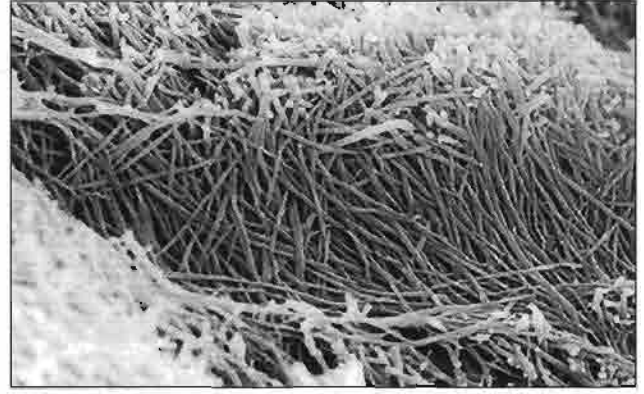


Fig 2-6b Higher magnification of the wall of the tubule in the predentin. No lamina limitans is present in predentin. Note the compact and woven arrangement of the collagen fibrils. These fibrils constitute the collagenous component of the intertubular dentin. (Adapted from Tabata et al⁴³ with permission from John Wiley & Sons. Original magnification $\times 31,000$.)

alization. The role of noncollagenous proteins in dentin formation has been the subject of recent reviews.^{67,68}

Dentin phosphophoryn is the major noncollagenous component of dentin. Immunocytochemical studies indicate that phosphophoryns are localized in small granules distinct from larger collagen-containing secretory granules. The DPPs are secreted from the odontoblastic process at the mineralization front.⁴⁹ Mineral crystal nucleation is attributed to DPP, a protein rich in aspartic acid and serine residues. Biochemical studies indicate that the DPPs are covalently linked to specific sites on the collagen fibrils of dentin.⁶⁹⁻⁷¹ The serine residues of DPPs are phosphorylated by casein kinase in the extracellular space prior to mineralization.^{72,73} Because of their many phosphate groups, and their capacity to bind calcium, DPPs create a template for calcium and phosphate concentration, and thereby drive crystal nucleation (see Fig 2-4).^{74,75}

An acidic phosphoprotein, DMP1 is localized to mature odontoblasts, cementoblasts, and osteoblasts.⁷⁶⁻⁷⁸ It is not expressed in the enamel organ and pulp. The precise role of DMP1 has yet to be identified. The gene coding for DMP1 has been localized to human chromosome 4q21, a region implicated in the autosomal-dominant form of dentinogenesis imperfecta type II.⁷⁹ Teeth affected by this disease are characterized by discolored and abnormally soft dentin and by fewer and irregular dentinal tubules.⁸⁰

Dentin sialoprotein is a sialic acid-rich glycoprotein that is expressed early in tooth development,

prior to degradation of the basement membrane. The mRNA for DSP has been detected in preameloblasts and preodontoblasts, suggesting that it may have a signaling role during ameloblast and odontoblast differentiation.^{81,82}

Both DSP and DPP are transcribed from a single mRNA, coded by a gene on human chromosome 4, and coexpressed during tooth development.^{83,84} Both proteins are expressed in preodontoblasts and odontoblasts throughout dentin matrix production. Preameloblasts also express DSP and DPP until enamel secretion, at which point mRNA for DSP and DPP is no longer detected in ameloblasts.

Additional glycoproteins, such as osteocalcin and thrombospondin 1, are found in dentin. Osteocalcin, a glycoprotein rich in glutamic acid, is present in odontoblasts and dentin matrix.^{31,85} In bone, osteocalcin is a chemotactic factor for osteoclasts. Thrombospondin 1 is present in high levels within predentin, especially near the mineralization front.⁸⁶ Thrombospondin 1 mRNA is localized in odontoblasts but not in the cells of the dental pulp.

Phospholipids

Cytochemical and autoradiographic studies have demonstrated the presence of phospholipids in predentin and dentin matrix. Because they are closely associated with hydroxyapatite crystals at the mineralization front, it has been speculated that they may have a role in mineral nucleation.⁸⁷

Growth factors

Bone morphogenetic proteins and TGF- β have been isolated from demineralized dentin matrix.⁸⁸ It has been suggested that they may trigger the differentiation of new odontoblasts during the induction of reparative dentin. Transforming growth factor β may be retained in the dentin matrix because of its ability to bind to decorin proteoglycan. Odontoblasts express high levels of TGF- β and its receptor.^{22,89} In addition, TGF- β promotes matrix production in most connective tissue cell types. Mutations in TGF- β genes lead to severe pulpal inflammation and attrition of occlusal surfaces.²³

Mineralization of Mantle and Circumpulpal Dentin

Two mechanisms for initiating crystal nucleation are responsible for mineralization of the dentin matrix: matrix vesicles in mantle dentin and collagen-phosphoryn complexes in circumpulpal dentin.

Matrix vesicles

Matrix vesicles in mantle dentin are similar to those first described in mineralizing cartilage.^{90,91} Matrix vesicles in mantle dentin are believed to bud from the tips of odontoblast cytoplasmic processes.

Matrix vesicles initiate mineralization by concentrating calcium and phosphate ions.⁹² Adenosine triphosphatase (ATPase) activity in matrix vesicle membranes may concentrate ions across the limiting membrane prior to nucleation.⁹³ As the ion concentration increases, hydroxyapatite crystallizes along the inner surface of the matrix vesicle membrane. Calcium-binding phospholipids in the limiting membrane may serve as templates for hydroxyapatite precipitation.

Elemental analysis of freeze-dried matrix vesicles in mantle predentin indicates that earliest mineral deposits appear as dotlike nuclei inside the vesicles.⁹⁴ Continued crystal growth leads to vesicle rupture, with release of hydroxyapatite crystals into the extracellular matrix. The newly formed crystals act as seeds for continued mineralization of mantle dentin matrix.

After mineralization is initiated, there appears to be no further need for matrix vesicles. Thus matrix vesicles do not participate in mineralization of late mantle dentin and circumpulpal dentin.⁹⁵ Matrix vesicles are also discussed in chapter 12.

Collagen-phosphoryn complexes

Mineralization of circumpulpal dentin is initiated by phosphoryns, independent of matrix vesicle activity.⁹⁶ Extracellular kinases have been identified in dental matrix.⁷³ These enzymes phosphorylate dentin phosphoryn. Dentin phosphoryns, linked to collagen fibrils, act as nucleators of hydroxyapatite crystals in late mantle dentin and circumpulpal dentin. The steric arrangement of negative charges on the phosphoryns creates a template for hydroxyapatite deposition.⁹⁷

Morphologic and histochemical studies of the predentin-dentin border in teeth that have been preserved by rapid pressure freezing and freeze substitution to avoid exposure to aqueous solvents have revealed a 0.5- to 2.0- μ m border zone within which mineral deposition occurs gradually.⁹⁸ In this zone, some proteoglycans are degraded and calcium is bound by phosphoryn and perhaps by phospholipid as well.

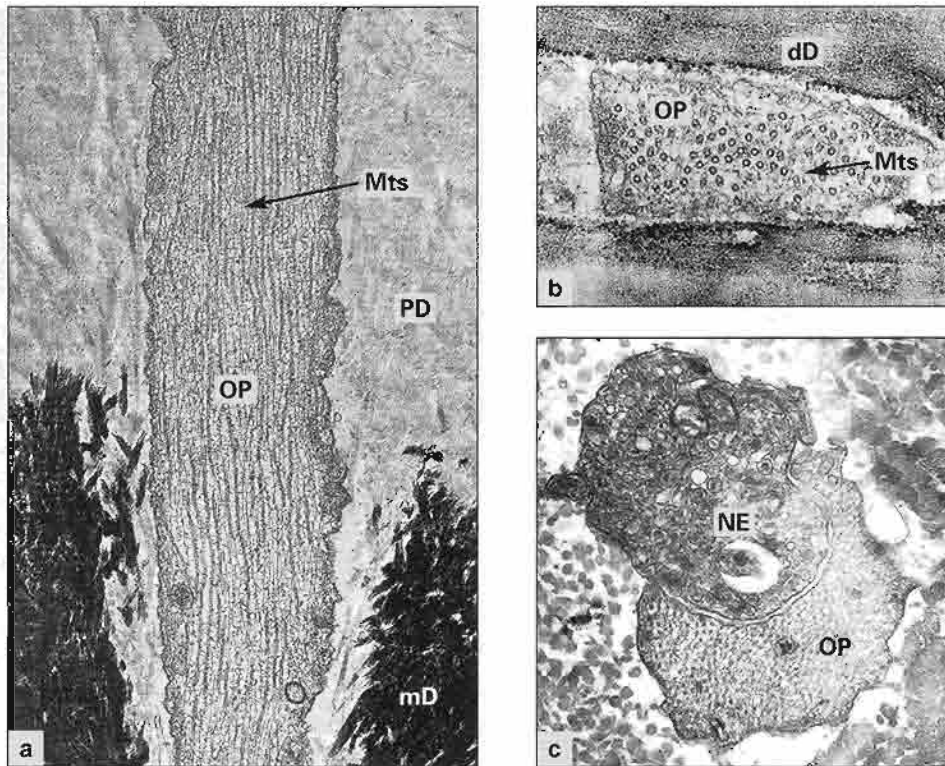
At the mineralization front in the zone of initial mineralization, dotlike mineral nuclei are aligned parallel to and superimposed on collagen fibrils.^{99,100} The mineral nuclei appear positioned over the hole regions of the collagen fibrils, suggesting that the DPPs are bound to collagen at those sites. Additional hydroxyapatite crystals have been located on the surface of the fibrils and in perifibrillar spaces. It has been suggested that dentin sialoproteins and proteoglycans act as nucleating agents for perifibrillar hydroxyapatite crystals. Collagen mineralization is discussed further in chapter 8.

Structure of the Odontoblastic Process and Dentinal Tubules

With progressive deposition of dentin matrix, the odontoblastic process lengthens and becomes embedded in mineralized tissue (Figs 2-2, 2-3, 2-6, and 2-7). The space occupied by the odontoblastic process is known as the *dentinal tubule*. The dentinal tubule extends from within the mantle dentin to the predentin.^{101,102}

Odontoblastic process

The cytoplasm of the odontoblastic process contains a rich network of microtubules and microfilaments, both of which are oriented parallel to its long axis (see Fig 2-7).^{50,103-105} The microtubules and microfila-



Figs 2-7a to 2-7c Electron micrographs of the odontoblastic process (OP) in longitudinal section (a) and cross section (b, c). The process contains a dense network of microtubules (Mts) and microfilaments, and is free of cytoplasmic organelles. Nerve endings (NE) can be found in close juxtaposition to the OP. (mD) Mineralized dentin; (PD) predentin; (dD) demineralized dentin. (Original magnification $\times 24,000$ [a], $\times 48,000$ [b], and $\times 30,000$ [c].)

ments form a network that runs the length of the odontoblastic process. Microtubules provide a substratum for granule translocation (discussed in chapter 3). The microfilaments (actin) provide a contractile mechanism that might enable the odontoblastic process to retract toward the pulp. It has been suggested that the odontoblastic process is retracted toward the cell body when exposed to noxious stimuli. This is an interesting idea that should be explored in new research.

When the odontoblastic process is viewed in cross section, it is apparent that microtubules and secretory granules are associated in a consistent pattern. The microtubules are distributed evenly around the circumference, about 30 to 40 nm from the granule membrane. Most secretory granules are secreted in the predentin or at the mineralization front.

Some secretory granules, however, are found in the odontoblastic process beyond the mineralization front, suggesting that collagen, proteoglycans, or

other constituents of the dentin matrix might be secreted from the distal regions of the process. Secretion of organic matrix from the distal parts of the process might be responsible for the formation of peritubular dentin and/or sclerosis of the tubules.

Numerous coated pits and coated vesicles indicative of membrane retrieval and receptor-mediated endocytosis are conspicuous elements of the odontoblastic process.¹⁰⁶

A sheath, rich in glycosaminoglycans, separates the process from the surface of the peritubular dentin (Figs 2-8 and 2-9).^{107,108} This sheath is similar to the lamina limitans found around osteocyte cell processes.¹⁰⁹ Thin cytoplasmic side branches, arising from the main odontoblastic process, pierce through the sheath and extend toward adjacent odontoblastic processes.¹¹⁰⁻¹¹² These small branches of the odontoblastic process contain only fine filaments.

Although it has been suggested that odontoblastic processes might communicate via side branches,

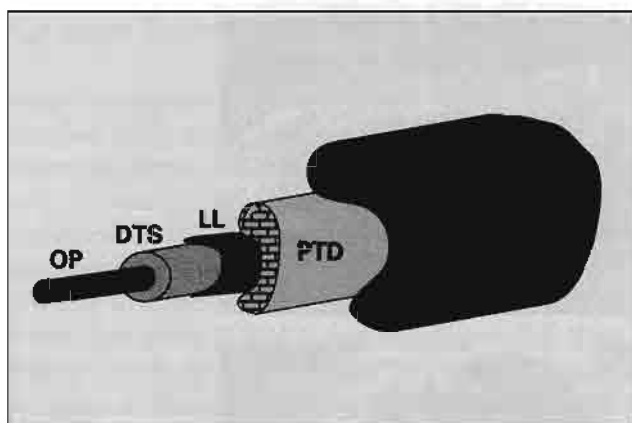


Fig 2-8 Components of tubular dentin. The odontoblastic process (OP) occupies the dentinal tubule space (DTS) and is surrounded by the lamina limitans (LL). (PTD) Peritubular dentin; (ITD) intertubular dentin.

there is no evidence that gap junctions are formed between adjacent side branches. If adjacent odontoblastic processes made gap junctional contacts via smaller side branches, their organization would be comparable to the canalicular cell processes of bone, whereby osteocytes intercommunicate through gap junctions.

Many investigators who have tried to determine the true length of the odontoblastic process by transmission electron microscopy have concluded that the process does not extend beyond the middle of the dentin.^{101,103,104,113} In contrast, most investigators who have examined fractured dentin surfaces by scanning electron microscopy have reported that the odontoblastic process extends out to the dentinoenamel junction.^{110,114-117} Dramatic scanning electron micrographs were obtained of dentin pretreated with hydrochloric acid to remove the mineral phase and with collagenase to remove the collagen fibrils of the organic matrix.¹¹⁷ A remarkable system of branching tubular structures, stretching from the predentin to the dentinoenamel junction, was revealed when these methods were used. The tubular structures were erroneously identified as odontoblastic processes.

The contrasting results obtained by routine transmission electron microscopy and scanning electron microscopy were resolved when it was shown that the structures presumed to represent odontoblastic processes could be removed by digestion with hyaluronidase.^{107,108,118} This was taken as proof that

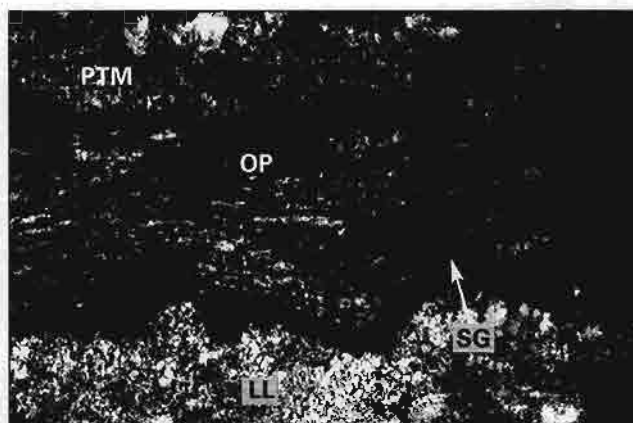


Fig 2-9 Odontoblastic process (OP) from middle region of the dentin viewed in longitudinal section. The lamina limitans (LL) is closely applied to the plasma membrane of the process. Demineralized peritubular matrix (PTM) abuts the LL. A secretory granule (SG) is present in the process. (Original magnification $\times 54,000$.)

the tubular structures, erroneously identified as odontoblastic processes, were in fact organic sheaths (lamina limitans) located between the odontoblastic process and the peritubular dentin. The true length of the odontoblastic process in mature dentin remained to be established.

Another approach to this problem was the use of fluorescein-labeled antitubulin as a method for identifying odontoblastic processes. In those studies, it was found that antitubulin was localized along the entire length of the dentinal tubule, suggesting, once again, that the odontoblastic process extended all the way to the dentinoenamel junction.¹¹⁹ An alternate explanation for the positive fluorescence might be that, following retraction or degradation of the odontoblastic process, tubulin might bind to the lamina limitans or to the walls of the dentinal tubules in sufficient amounts to give positive staining.

Confocal microscopic studies of odontoblasts infused with a fluorescent dye have shown that the odontoblastic process in fully developed teeth does not extend to the outer dentin.^{120,121} The longest processes were found in coronal dentin.

Another explanation put forth to explain the difficulty encountered in establishing the true length of the odontoblastic process is that the process might contract toward the cell body in response to noxious stimuli, such as the fixatives used to preserve cells prior to routine electron microscopy. This idea received support in studies of teeth that had been frozen rapidly to immobilize cytoplasmic structures

prior to chemical tissue fixation.¹²² When this approach was used, structures resembling odontoblastic processes were observed in dentin near the dentinoenamel junction. Additional research is needed to explore the interesting possibility that the odontoblastic process contracts in response to stimuli in its immediate environment.

Dental tubules

Dental tubules extend from the mantle dentin to the predentin, across the full thickness of circumpulpal dentin (see Fig 2-1). The distal end of the dental tubule branches extensively into fine secondary tubules that permeate the mantle dentin.^{123,124} Small side branches extend from tubule to tubule in the circumpulpal dentin.^{111,124} Dental tubules are wider toward the pulp and generally more concentrated in the region of the pulp horns.^{102,125}

Some dental tubules appear to be obliterated by nonmineralized collagen fibrils, while others are blocked with mineral.^{8,126,127} The physiology of dentin permeability is to a great degree a function of the patency of the tubules.^{108,128,129} Dental tubules contain serum proteins, including fibrinogen, albumin, and immunoglobulins.¹³⁰⁻¹³² These proteins are carried into the tubules in dental fluid, where they may become trapped in the lamina limitans or bound to the mineral phase of dentin. The flow of dental fluid increases following dental operative procedures and during pulpal inflammation.¹³³

Cariou dentin contains higher levels of immunoglobulins. It has been suggested that the high affinity of antibody binding to hydroxyapatite may serve as a protective reservoir of antibacterial immunoglobulin.¹³⁴

The first description of tubular "canals" in dentin was made by Leeuwenhoek in the 17th century. He fabricated simple microscopes capable of magnifications of no more than $\times 100$. With these rudimentary instruments, he amused his contemporaries by demonstrating microscopic canals in dentin, and animalcules (bacteria) in saliva. More than 300 years later, with microscopes capable of achieving magnifications of more than $\times 1,000,000$, there are still new frontiers to explore in the structure of dentin.

Formation of Intertubular and Peritubular Dentin

The greater bulk of the mineralized circumpulpal dentin is called *intertubular dentin* (see Fig 2-1). It is

formed by the mineralization of predentin. The matrix of intertubular dentin is rich in type I collagen fibrils. The uniform size and the arrangement of the collagen fibrils are best viewed in scanning electron micrographs (see Figs 2-6a and 2-6b).⁴³ Hydroxyapatite crystals, about 40 nm in length, are formed in and around the collagen fibrils of the intertubular dentin.

A second and minor component of circumpulpal dentin, the peritubular dentin, develops around the odontoblastic process (see Fig 2-1). The organic matrix of peritubular dentin is rich in glycosaminoglycans and relatively free of collagen fibrils. Bone sialoprotein and osteonectin have been localized in peritubular dentin. The hydroxyapatite crystals that develop in the peritubular dentin matrix are small in comparison to those that form in the intertubular dentin. Because of its small crystallites (large surface area) and the noncollagenous nature of its organic matrix, peritubular dentin is more susceptible to demineralization and degradation during the caries process.

Transport Across the Odontoblastic Layer

Over the course of several decades, there have been numerous contradictory reports on the transport of calcium and other substances into developing dentin. Some investigators have presented autoradiographic evidence of an intracellular (transcellular) pathway, while others have shown that ions move from pulp to dentin through a paracellular route between odontoblasts.

Increasing evidence supports a direct role for odontoblastic transport of ions into the predentin and dentin. The calcium ion activity in the fluid phase of predentin is two to three times higher than that in the pulp.¹³⁵ Current physiologic evidence suggests that an ionic gradient exists across the odontoblastic layer and that, under normal conditions, the paracellular pathway is closed. Under these conditions small molecules and ions would be forced to pass through the odontoblast cytoplasm to reach the dentin.

Recent studies have provided indirect support that calcium is transported into the dentin across the odontoblast cell membrane by a calcium ATPase pump, and by $\text{Na}^+\text{-Ca}^{++}$ antiports.^{136,137} L-type voltage-gated and agonist-gated calcium channels have been localized in odontoblasts by immunocytochemical methods.^{24,137,138} Calcium channel inhibitors (nifedipine and neomycin) have been shown to decrease the entry of calcium into the odontoblasts,

and eventually to reduce the level of calcium entry into the predentin. Secretory odontoblasts express high levels of inositol 1,4,5-triphosphate-regulated channel proteins. These channel proteins permit calcium flux from intracellular stores into the cytosol. Smutzer et al suggested that 1,4,5-triphosphate receptors might regulate calcium release for dentin mineralization.¹³⁹

The presence of ion concentration gradients across the odontoblastic layer appears to be at odds with the morphologic evidence that no zonula occludens is formed at the distal end of the odontoblast cell body. Furthermore, there is free diffusion of intravenously administered tracer molecules into predentin. Additional research is needed to identify the mechanisms responsible for generating and maintaining ion concentration gradients and for regulating solute movement across the odontoblast layer.

Innervation of Dentin and Mechanisms of Pain Sensation

Organization of the nerve supply

The nerve supply to the coronal pulp is especially rich, forming a subodontoblastic plexus of nerve fibers (plexus of Raschkow). Unmyelinated nerves from this plexus penetrate between the odontoblasts and enter the predentin. Although most unmyelinated nerves appear to terminate in the predentin, some enter the dentinal tubules. Nerve endings containing many small vesicles and mitochondria have been identified in close association with the odontoblast cell body and the odontoblastic process in dentin (Figs 2-7 and 2-10).¹⁴⁰⁻¹⁴³ The majority of these nerves are afferent somatosensory pain fibers.

Nerve growth factor and its receptor have been localized in relatively high amounts in the odontoblastic and subodontoblastic layers of the coronal pulp. The presence of nerve growth factor may be responsible for concentrating nerve endings in the vicinity of the odontoblasts. Experimental evidence has shown that nerves grow toward concentrations of nerve growth factor.

Because the cell processes of fibroblasts and odontoblasts can be confused with unmyelinated nerve fibers, special staining techniques are needed to accurately distinguish nerve fibers and their terminals. Protein gene product 9.5, a neuron-specific protein, has been used to identify pulpal nerve fibers at both the light and electron microscopic level (see Figs 2-10a to 2-10d).^{140,144} Fibers containing protein

gene product were present in both radicular and coronal predentin. Nerve endings in the predentin have also been identified by immunocytochemistry for calbindin, a calcium-binding protein found in high concentrations in nerve cells.¹⁴⁵ Tracer experiments with tritiated proline injected into the brainstem nuclei of the trigeminal nerve have provided convincing proof of a rich supply of sensory nerve terminals in the predentin and dentinal tubules.¹⁴⁶

Vasomotor nerves supply small arteries in the pulp, terminating in close apposition to arteriolar smooth muscle cells.¹⁴⁷ The nerve endings contain many dense-cored adrenergic synaptic vesicles.

Theories of dental pain

Although it is well established that most unmyelinated nerve endings in pulp and dentin are nociceptors, the exact mechanisms whereby noxious conditions are converted into dental pain stimuli have yet to be identified. The most widely held theory centers around fluid flow within dentinal tubules. The hydrodynamic theory of dental pain is based on the facts that fluid in the dentinal tubules is constrained by the rigid walls of the peritubular dentin and fluxes in temperature or in osmotic pressure produce rapid expansion and contraction of the dentinal fluid. The bulk flow of the dentinal fluid may distort nerve endings, thereby triggering nerve impulses. Experimental support for this theory is provided by the observation that pain occurs when heat or a substance that changes the osmotic pressure of the dentinal fluid is applied to cut dentin surfaces.

However, the hydrodynamic theory does not explain why some chemicals that do not alter osmotic pressure in dentin can still cause dental pain. Presumably these chemicals diffuse down their concentration gradients to act directly on nociceptive nerve endings in predentin. The hydrodynamic theory also fails to account for the rapidity of stimulus transduction, especially in relation to mechanoreceptor activity elicited from dentin (see chapter 9).

Despite the limitations of the hydrodynamic theory, the results of clinical and basic research on dentin sensitivity have shown that the patency of dentinal tubules is a significant factor in controlling the degree of stimulation of dentinal nerves.^{129,148-150} Recent investigations have shown that the dentinal tubules are filled with a fibrous hydrogel.¹²⁹ Although the hydrogel may limit bulk fluid flow, it still permits the diffusion of solutes down their concentration gradient. Dentin sensitivity can be reduced by obliteration of the tubules, either by the physiologic forma-

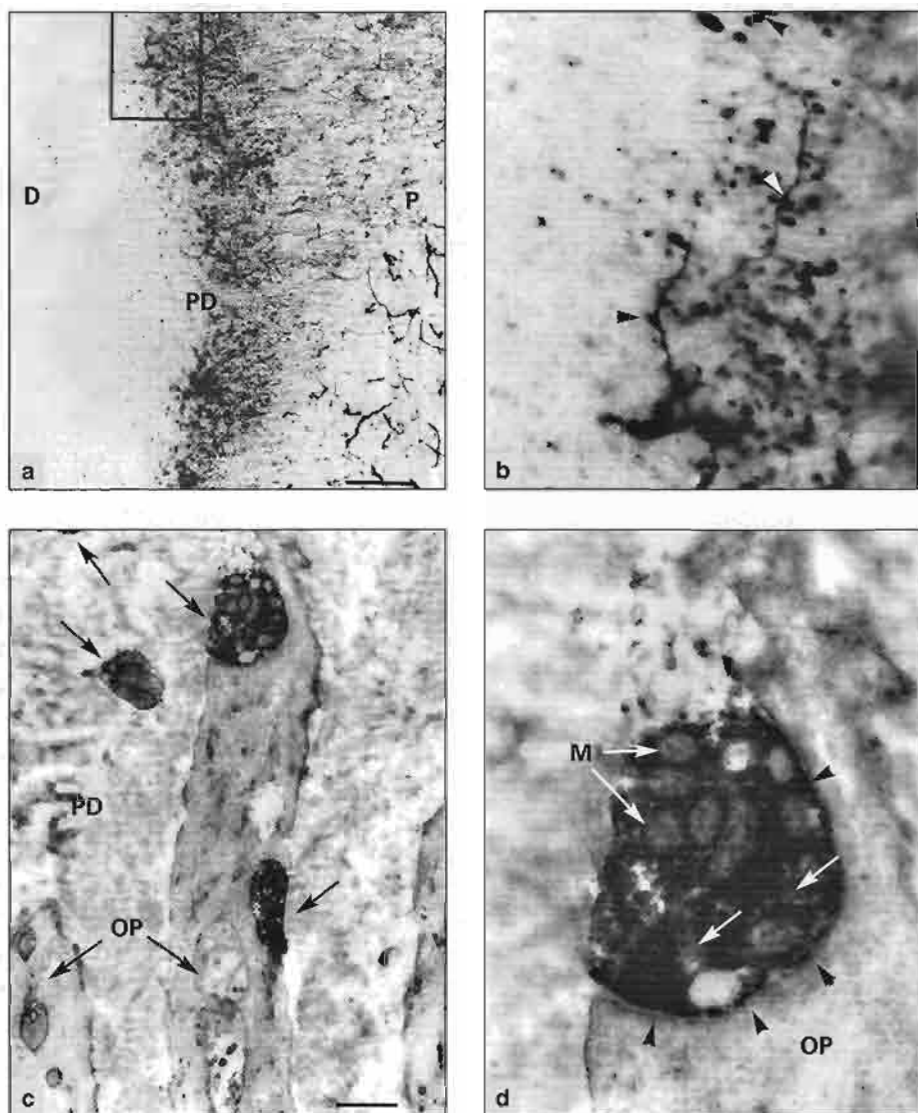
Figs 2-10a to 2-10d Nerve structure of a human premolar. (Human protein gene product [PGP] 9.5 antibody stain. Adapted from Maeda et al¹⁴⁰ with permission from Elsevier Science.)

Fig 2-10a Pulpodentinal region of human premolar stained with human PGP 9.5 antibody. A dense network of nerve endings can be seen in the predentin (PD). (D) Dentin; (P) pulp. (PGP 9.5 antibody stain. Original magnification $\times 70$.)

Fig 2-10b Inset area in Fig 2-10a at higher magnification. Nerve terminals are beadlike axonal swellings (arrowheads). (Original magnification $\times 700$.)

Fig 2-10c Electron immunocytochemistry. Nerve terminals (arrows) stained with PGP 9.5 antibody are juxtaposed to the odontoblastic processes (OP) in the predentin (PD). (Original magnification $\times 4,000$.)

Fig 2-10d Electron immunocytochemistry. The nerve terminals contain mitochondria (M) and many smooth vesicles (arrows). Although the plasma membranes of the nerve terminals are in close apposition to the plasma membrane of the odontoblastic process (OP) (arrowheads), no evidence of a synaptic structure is present. (Original magnification $\times 12,000$.)



tion of peritubular dentin and the intratubular deposition of collagen fibrils or by the clinical application of agents that cause mineral precipitation inside the tubules.

It has also been speculated that the odontoblast (and its process) might act as a transducer to convert noxious stimuli into nerve impulses. This concept is based on the notion that odontoblasts make gap junctions (electronic synapses) with adjacent nerves and that the flow of ions through these junctions, in response to changes in the odontoblasts, could lead to depolarization of somatosensory neu-

rons. Patch-clamp recordings made on segments of plasma membrane from isolated odontoblasts have demonstrated potassium and chloride channels and a resting membrane potential of about -40 to -50 mV.¹⁵¹ It has been suggested that mechanosensitive ion channels could lead to changes in ion conductance across the plasma membrane in response to hydrodynamic forces exerted on the odontoblastic process.¹⁵² Although gap junctions between odontoblasts and nerves have been reported, there is still no proof that odontoblasts communicate electrically with nerve endings.

A subpopulation of cells cultured from human dental pulp have voltage-gated sodium channels and other properties associated with neuronal satellite cells.¹⁵³ Whether these pulp cells originate from the odontoblastic layer and whether or not they have a role in pulpal somatosensation remains to be established. Additional discussion of dentinal and pulpal sensory mechanisms is contained in chapter 10.

It has also been suggested that dentinal nerves may have an effector function on odontoblasts. Indirect evidence for an effector activity includes the fact that many dentinal nerves contain the neuropeptide, calcitonin gene-related peptide (CGRP).¹⁵⁴ Evidence of protein secretion from dentinal nerves has also been reported.¹⁴² Recent studies of the presence of several exocytosis regulatory proteins (synapsin and synaptogamin) in dentinal nerve endings adds more support that dentinal nerves have an effector function in addition to their somatosensory afferent actions.¹⁵⁵

Many of the nerve endings in the odontoblastic layer and predentin contain substance P and CGRP.^{144,156-158} These neuropeptides may be involved in vascular dilation and neurogenic inflammation.¹⁵⁹⁻¹⁶⁰ Indirect evidence supports the idea that the release of neuropeptides from dental sensory nerve fibers is important in the recruitment of immunocompetent cells to the dental pulp.¹⁶¹ Experimental studies also suggest that these neuropeptides may promote dentinogenesis.^{162,163}

The potential for development of neurogenic inflammation in pulp is supported by the demonstration that coronal and apical pulp contain CGRP-positive nerves in association with blood vessels and within the connective tissue stroma.^{159,160,164} Some CGRP-positive nerve fibers are found in the subodontoblastic layer. Sprouting of CGRP-positive nerve endings occurs following dental injury.^{165,166} The release of CGRP increases vascular permeability of pulpal blood vessels.¹⁶⁷ Recent studies have demonstrated excitatory amino acid receptors in bovine dental pulp.¹⁶⁸ Activation of excitatory amino acid receptors leads to the release of CGRP in pulp.

Supply of Blood to the Pulp

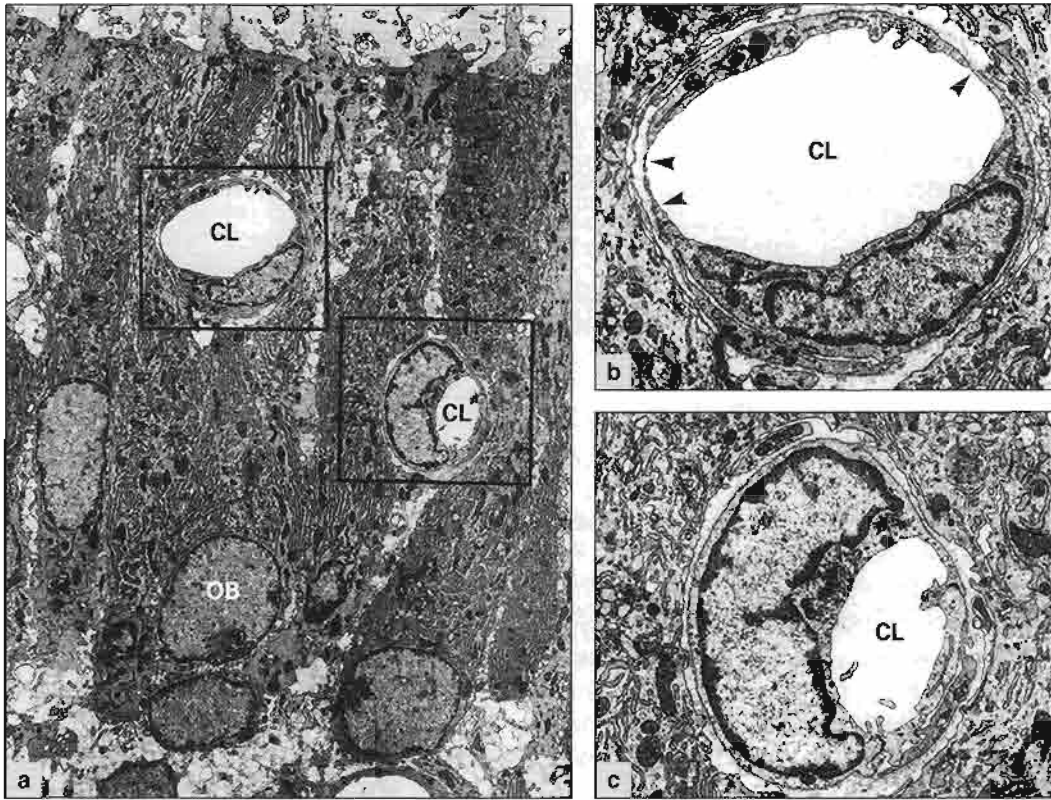
Blood vessels enter the tooth through the apical foramen and course coronally in the midregion of the radicular pulp. The largest arteries have a muscular coat of three to six layers of smooth-muscle cells. The outer adventitia is rather inconspicuous, because it blends gradually with the pulpal connective

tissue. The endothelial cells that line the arteries often appear to bulge into the lumen. The accompanying veins have one to two layers of smooth-muscle cells and a wider lumen. As the major vessels course through the radicular pulp, they give off peripheral branches that arborize to form a rich capillary network associated with the odontoblastic layer.^{169,170} The greatest degree of branching occurs in the coronal pulp, especially below the cusps.

The endothelial lining is of the continuous type, except for the fenestrated capillaries that are adjacent to the predentin. The cell-to-cell junctions of the endothelium are characterized by adherens junctions and overlapping cell processes. Numerous pynocytotic vesicles are present on the luminal and abluminal surfaces of the endothelium. The luminal surface of capillaries and venules contain many cytoplasmic processes. Spindle-shaped pericytes contain moderately high numbers of cytoplasmic filaments and are spaced apart as single cells in close contact with the basal lamina of the endothelial cells. The pericytes serve as stem cells capable of multipotential differentiation. Recent studies have shown that a population of dendritic cells is associated with the major pulpal blood vessels.^{171,172} These cells are specialized for phagocytosis, processing, and presentation of antigens.

Blood is supplied to the odontoblastic layer by capillaries that are in close apposition to the odontoblast cell bodies and the predentin (Figs 2-11a to 2-11c).¹⁷³⁻¹⁷⁵ Individual capillaries penetrate the intercellular spaces between the odontoblasts to form the predentin capillary plexus. The predentin capillary plexus reaches a peak of development coincident to the most active phase of dentin formation. Fenestrated endothelial linings have been reported in capillaries located close to the predentin.¹⁶⁹ The close proximity of thin-walled capillaries to the odontoblasts and the predentin suggests that there is a high requirement for oxygen, ions, and metabolites during the rapid phase of dentin formation. When dentin formation is completed, the predentin capillary plexus is no longer present. At this stage, nutrients reach the odontoblasts from the subodontoblastic plexus.

Reparative dentinogenesis is preceded by angiogenesis. Several angiogenic growth factors (platelet-derived growth factor, vascular endothelial growth factor, and fibroblast growth factor) have been isolated from dentin matrix.¹⁷⁶ It has been proposed that angiogenic growth factors are released during dentin degradation, thereby stimulating the development of new blood vessels in the zone of injury.¹⁷⁶



Figs 2-11a to 2-11c Blood supply to the pulp. (Adapted from Ohshima and Yoshida¹⁶⁹ with permission from Springer-Verlag.) (a) Electron micrograph illustrating the presence of capillaries (CL) in the odontoblastic (OB) layer. (Original magnification $\times 3,000$.) (b) Left inset area in Fig 2-11a at higher magnification. Capillaries (CL) are generally of the fenestrated type near the predentin (arrowheads). (Original magnification $\times 8,000$.) (c) Right inset area in Fig 2-11a at higher magnification. Capillaries (CL) are generally of the continuous type nearer to the pulp. (Original magnification $\times 8,000$.)

Cells and Extracellular Matrix of the Dental Pulp

The dental pulp is a connective tissue derived from the proliferation and differentiation of the cells of the dental papilla. In its developmental stage, the dental pulp contains a relatively high content of glycosaminoglycans and sparsely distributed, fine collagen fibrils (types I and III).^{177,178} Initially, the network of collagen fibers is composed mostly of argyrophilic reticular fibers rich in type III collagen. As the pulp matures, the synthetic capacity of pulpal fibroblasts increases, and more collagen bundles of type I collagen are formed. Despite the increased amount of type I collagen, the mature pulp continues to have an unusually high content of type III collagen.¹⁷⁹ Al-

though the numbers of collagen fibers continue to increase with age, the pulp maintains its appearance of a loose connective tissue. Collagen fibers are concentrated to form supporting elements for blood vessels and nerve trunks that course from the root apex to the coronal pulp chamber.

The pulp contains a relatively large concentration of glycosaminoglycans and proteoglycans.^{180,181} Versican, a chondroitin-6-sulfate-rich proteoglycan, has been detected in high concentrations in peripheral pulp.¹⁸² Fibroblasts are distributed evenly throughout the middle regions of the pulp and concentrated beneath the odontoblastic layer in the coronal pulp of erupted teeth to form a cell-rich zone. The cell-rich zone also contains numerous major histocompatibility complex-positive dendritic cells that have an increased capacity for capturing and processing anti-

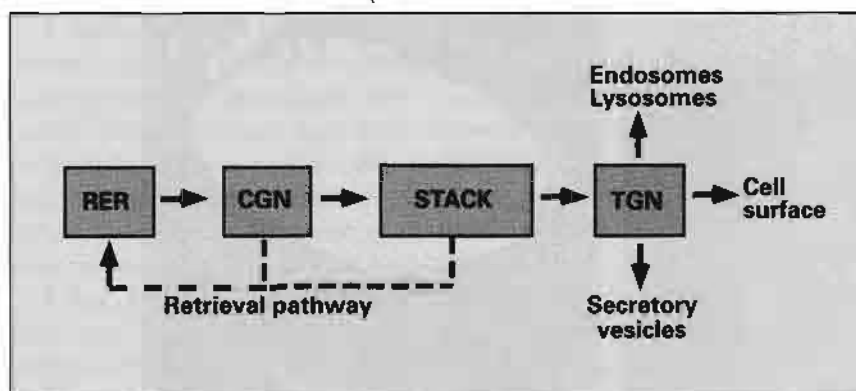


Fig 2-12 Secretory pathway from the rough endoplasmic reticulum (RER) to the cis-Golgi network (CGN), across the Golgi stacks, and into the trans-Golgi network (TGN), where proteins are directed to appropriate destinations. (arrows) Unidirectional anterograde vesicular transport. (dashed lines) Retrograde pathways used to retrieve membrane and proteins that have escaped from the RER. (Adapted from Rothman and Orci¹⁸⁷ with permission from MacMillan Publishers.)

gens.¹⁷² These cells have numerous cell processes that make contact with odontoblasts and nerves.¹⁸³ Dendritic cells are part of the surveillance arm of the immune system (see chapter 13). Because the cell-rich zone makes its appearance after the tooth has erupted into the oral cavity and is limited in its extent to the coronal pulp (excluding the floor of the coronal pulp), it is believed to form as a defensive response to external stimulation.

Dental pulp cells respond to a variety of growth factors.¹⁸⁴ Deoxyribonucleic acid synthesis in human dental pulp cells is stimulated by basic fibroblast growth factor and platelet-derived growth factor and is inhibited by interleukin 1 β . Transforming growth factor β stimulates the synthesis of collagen and fibronectin in cultures of pulp cells.¹⁸⁴ Vitamin D stimulates pulp fibroblasts to express osteopontin, a phosphoprotein typically found in bone.¹⁸⁵

Basic Science Correlation: The Secretory Pathway

During the 1960s and 1970s, the ultrastructure of the RER-Golgi system was characterized, and the morphologic aspects of a secretory pathway were established. It is now known that proteins destined to be exported from the cell, or to lysosomes and endosomes, are synthesized in the rough endoplasmic reticulum and transported to the Golgi complex.¹⁸⁶ In the Golgi complex, proteins are posttranslationally modified, sorted, and packaged for further transport to their ultimate destination, whether it be a secretory granule, a primary lysosome, or the cell membrane.

No specific signal recognition event appears to be required for the transport of proteins from the RER to the Golgi apparatus. The only prerequisite is that the proteins undergo correct three-dimensional folding

within the RER. Transport vesicles destined for the Golgi apparatus develop from smooth-membrane segments of the RER, called *transitional elements*.

The Golgi complex is subdivided into a cis-Golgi network, Golgi stacks, and a trans-Golgi network (Fig 2-12).¹⁸⁷ The cis-Golgi network acts as a quality control gate, preventing the transport of defective proteins through the Golgi complex to the cell surface and/or secretion into the extracellular space. The small percentage of RER-resident proteins that escape during the formation of transport vesicles are recognized in the cis-Golgi network by their lysine-aspartic acid-glutamic acid-leucine amino acid sequence and are returned to the RER in a retrograde vesicular pathway (see Fig 2-12).¹⁸⁸ Retrograde traffic also returns membrane lipids to the RER compartment. Transport from the RER to the Golgi apparatus requires microtubules. However, the retrograde pathway from the Golgi apparatus back to the RER does not depend on an intact microtubular network.

Secretory and cell membrane proteins undergo successive compartment-specific reactions during their transit through the Golgi stacks. Glycosyltransferase and glycosidases contained in the Golgi cisternae sequentially decorate the peptide backbone of the protein by the addition of carbohydrate side chains. These posttranslational modifications involve the addition of oligosaccharides by nitrogen-linkage to asparagine, and/or oxygen-linkage at serine and threonine residues. Formation of oxygen-linked glycans involves a two-step process consisting of the addition of *N*-acetyl-galactosamine, followed by the addition of galactose and sialic acid (*N*-acetyl-neuraminic acid). Studies have shown that the addition of *N*-acetyl-galactosamine occurs in transitional elements of the RER, while the addition of galactose and sialic acid occurs in the most mature cisternae of the Golgi apparatus.

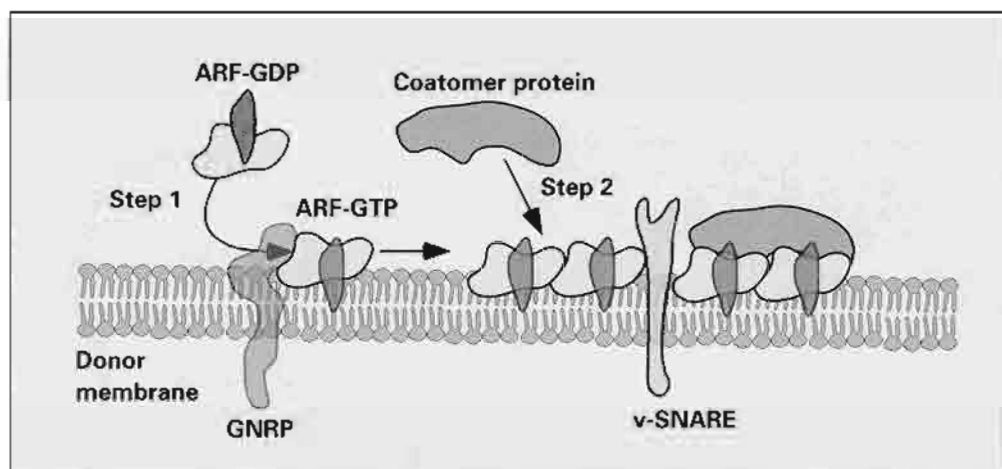


Fig 2-13 Formation of a coatamer-coated membrane. The first step involves the insertion of guanine nucleotide-releasing protein (GNRP) into the membrane of the donor compartment. In the second step, GNRP reacts with adenosine diphosphate ribosylation factor (ARF), converting ARF-guanosine diphosphate (GDP) to ARF-guanosine triphosphate (GTP). Attached to the donor membrane, ARF-GTP is then able to bind coatamer proteins. As more coatamer proteins are bound to the site, a vesicle will start to form from the donor compartment by a budding process. Soluble *N*-ethylmaleimide-sensitive fusion attachment protein receptors (SNAREs) project from the surface of the transport vesicle (v-SNARE).

The two-way traffic of vesicles from the RER to the Golgi complex, and from the Golgi complex to the cell membrane, requires numerous regulatory mechanisms.¹⁸⁹ The complex machinery for sorting proteins and controlling vesicular traffic inside the cell began to be deciphered in the 1980s and was accelerated by the advent of newly discovered molecular biology techniques.¹⁹⁰ Cell biologists view the Golgi complex as a dynamic system of membrane-bound compartments whose function requires constant intercompartmental vesicular exchange. Movement of substances from the RER to the cis-Golgi network, between Golgi stacks, and from the trans-Golgi network to the final target membrane is carried out in small transport vesicles that bud from surfaces of the donor compartment.^{191,192} A great deal of research is being focused on identifying the molecular nature of the sorting, docking, and fusion events needed for this operation.

The budding process requires the recruitment and attachment of specific coat proteins (coatamers) on the parent cisternal membrane to form a mechanochemical "patch" capable of deforming the membrane into a separate vesicle.¹⁹³⁻¹⁹⁵ As the vesicle forms, it concentrates a microscopic sample of specific cargo proteins from the cisternal fluid. Coatamer recruitment requires ATP, Ca^{2+} , guanosine triphosphate (GTP), and several cytosolic proteins.¹⁹⁶

In the first step of the process (Fig 2-13), a transmembrane protein in the donor membrane, guanine nucleotide-releasing protein (GNRP), interacts with a cytosolic GTP-binding protein called *adenosine diphosphate ribosylation factor* (ARF). In the cytosol, ARF is in its guanosine diphosphate (GDP)-bound state (ARF-GDP). When ARF-GDP interacts with GNRP, GDP is released and GTP is bound in its place. Subsequently, ARF-GTP undergoes conformational change, exposing a fatty acid chain that anchors ARF-GTP to the donor membrane.

In step two, segments of the membrane covered by ARF-GTP favor the recruitment and attachment of coatamer proteins (see Fig 2-13). In mechanisms yet to be clarified, the coatamer-coated membrane is deformed and pinched off to form a coatamer-coated transport vesicle (Fig 2-14). Transport vesicles retain their coatamer coats until they begin docking to the appropriate target membrane.

Sorting products to their appropriate destinations requires specific signals to control the docking of transport vesicles with the correct target compartment. This is accomplished by transmembrane proteins that act as surface markers. The transmembrane interacting proteins are soluble *N*-ethylmaleimide-sensitive fusion attachment protein receptors (SNAREs). Terrian and White reviewed the evolution of SNARE proteins and their role in traffic regulation (see Fig 2-14).¹⁹⁷ Spe-

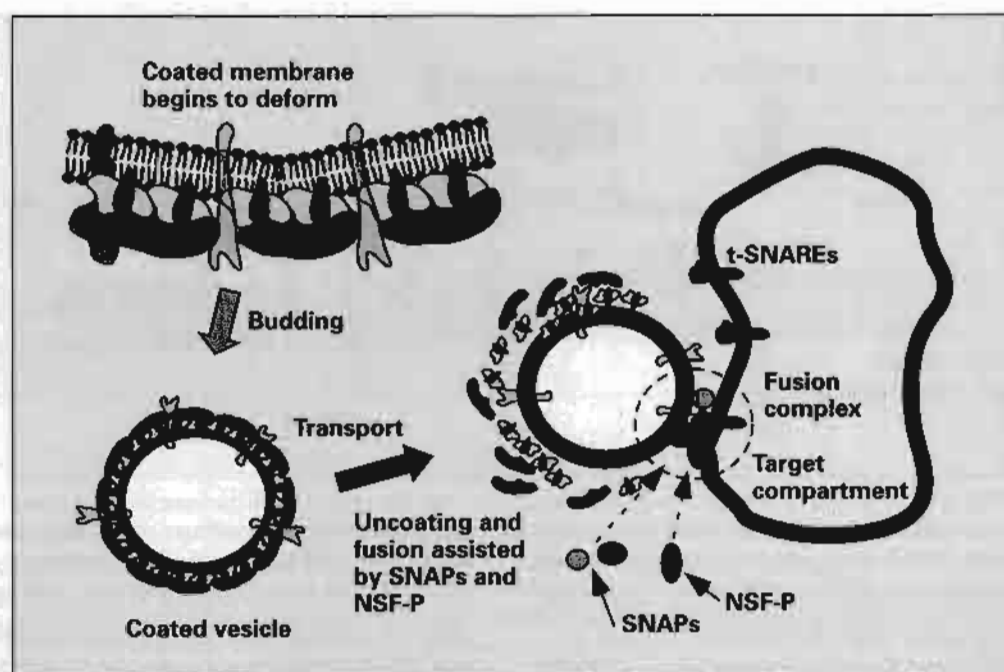


Fig 2-14 Formation, docking, and fusion of the transport vesicle. The coatamer-coated vesicle, with soluble *N*-ethylmaleimide-sensitive fusion attachment protein receptor (v-SNARE) molecules exposed beyond the coatamer coating, is available for binding (step 2, docking reaction) to the appropriate target membrane SNARE (t-SNARE) molecules. During the docking reaction, adenosine diphosphate ribosylation factor-guanosine triphosphate is hydrolyzed to adenosine diphosphate ribosylation factor-guanosine diphosphate and disassociates from the vesicle membrane. Coatamer proteins are also released. The attachment of v-SNARE to t-SNARE is monitored and stabilized by a second type of guanosine triphosphate, a Rab-GTP molecule present in the donor vesicle membrane. Fusion of the transport vesicle membrane to the target (step 3) is induced by a protein complex that includes *N*-ethylmaleimide-sensitive fusion protein (NSF-P) and soluble NSF attachment proteins (SNAPs).

cific surface markers (t-SNAREs) have been identified in the membranes of the RER, Golgi complex, endosomes, and plasma membrane.

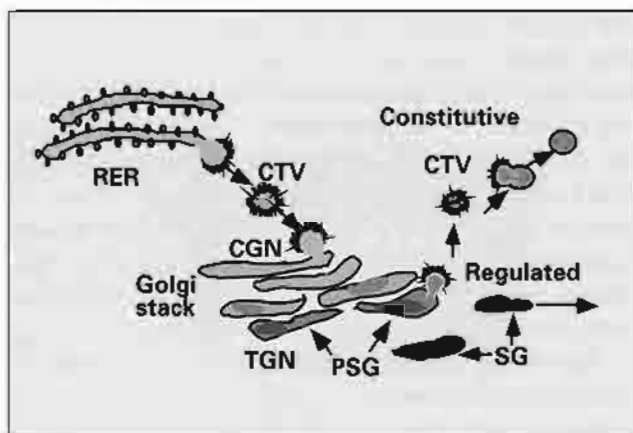
Docking of the transport vesicle to the target membrane occurs when vesicle SNAREs (v-SNAREs) bind to their "target" membranes (t-SNAREs) (see Fig 2-14). Rab-GTP, a second type of monomeric guanosine triphosphatase, present in the vesicle membrane, functions as a monitor and stabilizer of the fit between the two types of SNARE molecules. A guanosine triphosphatase-activating protein in the target membrane causes ARF-GTP to hydrolyze GTP to GDP. In its GDP-binding state, ARF retracts its fatty acid anchor and detaches from the vesicle membrane. Simultaneously, the ARF-coatamer complex disassembles.

For membrane fusion to occur, special fusion proteins are required to displace water molecules and to overcome the electrostatic repulsive forces between the two closely juxtaposed lipid membranes.¹⁹⁸ The

space between the adjacent membranes must be reduced to less than 1.5 nm. The v-SNARE to t-SNARE receptor-ligand docking reaction between the transport vesicle and its target membrane recruits fusion proteins to the site of attachment. *N*-ethylmaleimide-sensitive fusion (NSF) protein and soluble NSF attachment proteins (SNAPs) have been shown to carry out fusions in eukaryotic cells by interacting with SNAREs (see Fig 2-14). Conformational changes in the fusion protein, driven by ATP, destabilize the lipid membranes, leading to the formation of a fusion pore that rapidly expands to permit total fusion of the two membranes. Exactly how this machinery is assembled and how it functions during the fusion event is still speculative.

Application of this new knowledge of the regulation of cytoplasmic traffic must be applied to future studies of the odontoblastic Golgi complex to gain a clearer understanding of the secretion and retrieval of dentin matrix components.

Fig 2-15 Odontoblast secretory pathway. Intermediate coated transport vesicles (CTV) bud from the rough endoplasmic reticulum (RER) and migrate to the cis-Golgi network (CGN), where they fuse with the outermost cisternae of the Golgi stack. Pre-secretory granules (PSG) form part of the trans-Golgi network (TGN). Concentration of the secretory product occurs by aggregation of proteins inside the PSGs and by the removal of fluid and membrane via budding of small vesicles. Vesicles containing membrane proteins and lipids are secreted in the constitutive pathway. Matrix secretory granules (SG) are transported via the regulated pathway, along microtubules, to the odontoblastic process.



From the trans-Golgi network, there are two basic pathways of secretion: the regulated pathway and the constitutive pathway.^{199,200} In the regulated pathway, secretory product is stored in vesicles or granules until secretion is triggered by an appropriate signal. Secretory granule formation involves condensation of the secretory product from larger condensing vacuoles (presecretory granules) considered to be part of the trans-Golgi network (see Fig 2-13).²⁰¹ Budding of membrane from the condensing vacuole continues, until a smaller and denser secretory granule is formed. In the constitutive pathway, products are exported immediately after they are packaged in the Golgi apparatus.

Regulated secretion requires an intact microtubular system to transport granules to a specific region of the cell surface, usually the apical end of the cell. Constitutive secretion does not appear to be dependent on microtubules and may occur from many regions of the cell surface.

Microtubules form a radiating network, extending from the centrosome outward to the cell periphery. This network provides a structural pathway for the translocation of secretory granules. The energy source for granule transport is derived from the hydrolysis of ATP. Enzymatic motor proteins (mechanochemical ATPases) associated with the granule membrane hydrolyse ATP molecules when activated by contact with the microtubules.

Cyclical attachment and detachment produces movement of the granule along the length of the microtubule. The mechanochemical ATPase responsible for anterograde movement of secretory granules is a member of the kinesin family of motor proteins. Substances that interfere with microtubule assembly, such as antimitotic (antispindle) agents, and

substances that deplete ATP or stop its production produce abnormalities in deposition of dentin, enamel, and bone matrices.

Microtubule-directed transport delivers secretory granules to the periphery of the secretory pole of the cell. At that point, further transport toward the plasma membrane is dependent on myosin. The final approach and fusion is both nonmicrotubule and nonmyosin dependent.²⁰² Discussion of microtubules is continued in chapter 3.

A proposed pathway for secretion in the odontoblast, based on ultrastructural studies of the odontoblast and the current theories of Golgi organization, is outlined in Fig 2-15. Additional studies of odontoblast structure and function are needed to determine whether there are multiple forms of secretory granules in odontoblasts and to identify the signals that direct secretory granule discharge.

Clinical Correlations

Secondary, tertiary (reactive), and reparative dentin

Odontoblasts are nondividing cells with a long life span. During tooth development, they produce primary dentin at the rate of about 4 to 8 μm per day. Once the crown is completed and the apical length of the root has been established, odontoblasts produce secondary dentin at 1 to 2 μm per day. Histologic studies of human teeth have shown that all teeth contain secondary dentin. It is deposited throughout life as long as the pulp remains vital.

Although there are no reliable data on the estimated longevity of individual odontoblasts, the con-

tinued slow deposition of secondary dentin suggests that odontoblasts are long-lived cells. Odontoblasts lose nearly half of their RER and Golgi profiles following formation of primary dentin.²⁰³ Biochemical studies indicate that, on completion of primary dentin, there is an 80% reduction in alkaline phosphatase activity at the predentin-odontoblast region, and a concomitant reduction of ATPase activity in the odontoblasts. This reduced metabolic function is consistent with a slow production of secondary dentin.

Secondary dentin is characterized by a regular arrangement of dentinal tubules, usually in direct continuity with those of the primary dentin. Microhardness measurements indicate that secondary dentin is about 30% to 40% softer than primary dentin. The biochemical and matrix factors responsible for the decrease in mineral content have not been identified.

Tertiary or reactive dentin is produced in response to nonlethal irritation of the odontoblasts. Once activated, either by a slowly progressing carious lesion, dental abrasion, or restorative dentistry procedure, odontoblasts resume deposition of dentin at rates that approach those measured for formation of primary dentin. Reactive dentin is deposited subjacent to the area of injury as a protective barrier for the pulp.

Reactive dentin does not have the well-organized histologic structure of primary or secondary dentin. The dentinal tubules are fewer and less likely to be neatly parallel to each other. A calciotraumatic line is commonly found to separate the secondary dentin from the reactive dentin. Excessive formation of reactive dentin in the root portion of the pulp can lead to varying degrees of pulp canal obliteration, a condition that complicates pulp canal therapy.

Recent studies of superficial carious lesions, where the zone of demineralization had not reached the dentinoenamel junction, demonstrated increased deposition of peritubular dentin and a decreased width of the predentin.²⁰⁴ With progression of the lesion to the dentinoenamel junction, the predentin grew wider than control predentin as deposition of collagen increased. Cell proliferation in the subodontoblastic layer accompanied changes in activity of the odontoblasts.

It was suggested that in early enamel caries, the odontoblasts respond to stimuli transmitted along partially demineralized enamel rods and the dentinal tubules.²⁰⁴ When the caries process involves dentin, fibronectin and a 165-kDa fibronectin-binding protein are deposited on the surface of the odontoblastic process and along the walls of the dentinal tubules.²⁰⁵ It was suggested that fibronectin

and 165-kDa proteins regulate reactive dentinogenesis, perhaps by playing a signaling role similar to that which occurs during initial odontoblast differentiation.

If the injury is severe, as in rapidly advancing dental caries or in a dental operative procedure producing excessive heat, the odontoblasts undergo necrosis. In this case, repair (the formation of reparative dentin) must await the differentiation of new odontoblast-like cells from precursors, either from the cell-rich zone or from deeper regions of the pulp.²⁰⁶ The presence of a fibronectin-rich surface, permitting adhesion of pulp cells, appears essential to the differentiation of odontoblast-like cells.²⁰⁷

Replacement odontoblasts are generally produced in fewer numbers than the original complement. The reparative dentin that they deposit is characterized by irregular tubules. Under less favorable conditions, new odontoblasts may fail to differentiate, and repair is carried out by fibroblastic cells that deposit a fibrodentin type of matrix. In either case, the process requires several weeks.

Recent experiments have shown that certain growth factors, namely bone morphogenetic protein (BMP-2, BMP-4, and BMP-7), TGF- β 1, and components of dentin matrix, stimulate the development of the odontoblast phenotype and the expression of type I collagen and osteocalcin in pulp cells.²⁰⁸⁻²¹⁰ Cultured human pulp fibroblasts express BMPs and BMP receptor.^{209,211,212} In the presence of fibronectin, TGF- β 1 and BMP-2 trigger odontoblast development from embryonic dental papilla cells.^{89,213} It has been proposed that these growth factors have a regulatory role in the initiation of reparative dentin by activating the differentiation of new odontoblasts.²¹⁴⁻²¹⁶

The potential value of BMPs as pulp-capping agents is under investigation by several research groups. Recombinant human BMP-2, BMP-4, and BMP-7 have been incorporated in pulp-capping preparations applied to the pulps of dogs and monkeys. Significant increases in reparative dentin were noted after several months in teeth capped with BMP preparations.^{210,217-219} High-molecular weight hyaluronic acid also promotes formation of reparative dentin in amputated dental pulp.²²⁰

Sclerotic dentin

Empty dentinal tubules result from either the physiologic retraction of the odontoblastic process or from the death of the odontoblasts. These tubules appear as dark bands (dead tracts) in ground mineralized sections when viewed under transmitted light. Open

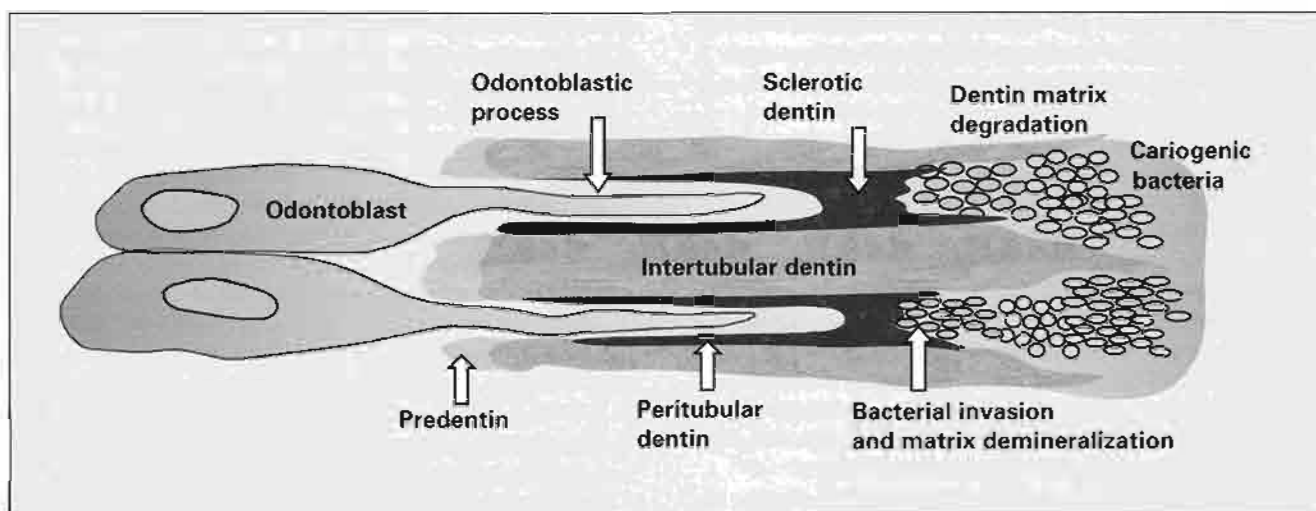


Fig 2-16 In a caries lesion, cariogenic bacteria invade the dentinal tubules, demineralizing sclerotic and peritubular dentin in the process. Intertubular dentin is slower to degrade because of its dense collagenous matrix and larger hydroxyapatite crystals.

dentinal tubules, especially at the cervical region, often lead to dental hypersensitivity. Occlusion of the tubules by precipitation of calcium salts or with composite resin reduces the flow of fluid and decreases the sensation of pain.²²¹⁻²²³

During mild irritation, dentinal tubules may become obliterated by mineral deposition, a process known as *dentinal sclerosis* (Fig 2-16). Some investigators have suggested that continued or excessive deposition of peritubular dentin is the basis of dentinal sclerosis. Sclerotic dentin is usually present under chronic carious lesions, dental restorations, and areas of attrition. It has been suggested that sclerosis of dentinal tubules is a defense mechanism for protection of the pulp. In ground sections, sclerotic dentin appears translucent, blending in closely with adjacent mineralized intertubular dentin. The distal portion of the odontoblastic process may become mineralized in carious dentin.¹⁰³

In the caries process, dentin demineralization begins when the enamel lesion reaches the dentino-enamel junction. The caries process does not spread preferentially along the dentinoenamel junction but rather progresses into the dentin along the dentinal tubules.²²⁴ In sclerotic tubules, bacteria advance more slowly because they must remove hydroxyapatite crystals by acid dissolution. Nevertheless, because of the small crystallites (large surface area for exchange) in sclerotic and peritubular dentin, and

the absence of a collagen fibril matrix, bacteria are able to advance preferentially along the tubules (see Fig 2-16). Destruction of the collagenous matrix of intertubular dentin proceeds more slowly, because proteolytic enzymes must gain access to and degrade the collagen matrix after it has been demineralized. Bacterial invasion of the dentinal tubules is a complex process involving bacterial adhesions to extracellular matrix molecules, proteolytic enzymes, and the ability of bacteria to survive in an environment of limited oxygen and nutrients.²²⁵

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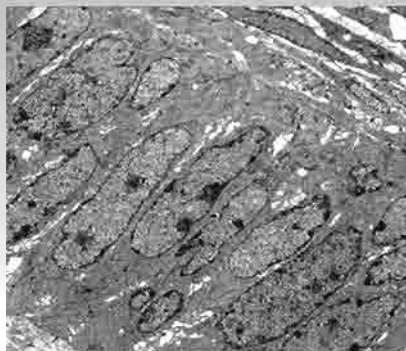
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Chapter 3

Enamel



Differentiation of the Enamel Organ

During the early stage of tooth formation, the enamel organ consists of the outer enamel epithelium (OEE), the cells of the stellate reticulum (SR), the stratum intermedium (SI), and the inner enamel epithelium (IEE) (see Fig 1-6). The cells of the outer enamel epithelium are generally cuboidal. They attach by hemidesmosomes to a basal lamina separating them from the adjacent dental sac, a connective tissue of ectomesenchymal origin. Cytoplasmic organelles in the OEE include a moderate number of mitochondria, a small number of cisterns of rough endoplasmic reticulum (RER) and smooth endoplasmic reticulum, and a poorly developed Golgi complex. The presence of coated vesicles in the peripheral cytoplasm and along the plasma membrane facing the basal lamina suggests that the OEE is involved in specific endocytosis of extracellular substances.

Soon after the onset of enamel formation, the OEE becomes convoluted by indentations of highly vascularized connective tissue. This structural change becomes pronounced during enamel maturation, when the OEE, the SR, and the SI form the papillary layer to increase the surface area between the enamel organ and the adjacent blood supply. This change is pronounced in the continuously develop-

ing incisor of the rat, the most thoroughly investigated model of tooth development.

The cells of the SR have a compact cell body with many long folds of cytoplasm that contact and partly overlap similar cytoplasmic folds from adjacent cells. Desmosomes and gap junctions are formed at points of cell-to-cell contact. These folds of cytoplasm line wide extracellular spaces rich in water-binding glycosaminoglycans.

The soft, jellylike consistency of the enamel organ is due to the hydration of the SR glycosaminoglycans. This property is believed to be important in equalizing pressure generated by cell proliferation and matrix secretion in the dental papilla. It has been suggested that, if external tissue-generated forces at the interface between the preameloblasts and the preodontoblasts are eliminated, the three-dimensional outline of the future dentinoenamel junction, and ultimately the shape of the crown of the tooth, can be molded solely by the forces of cell proliferation in both the IEE and the underlying preodontoblasts of the dental papilla. With the onset of ameloblast differentiation, the formation of a terminal web in the IEE, coupled with the assembly of a basement membrane beneath the IEE, stabilizes the shape of the future dentinoenamel junction.

The cells of the SI form a compact zone, one to two cellular layers deep, between the SR and the IEE (Figs 3-1 and 3-2). The intercellular spaces between

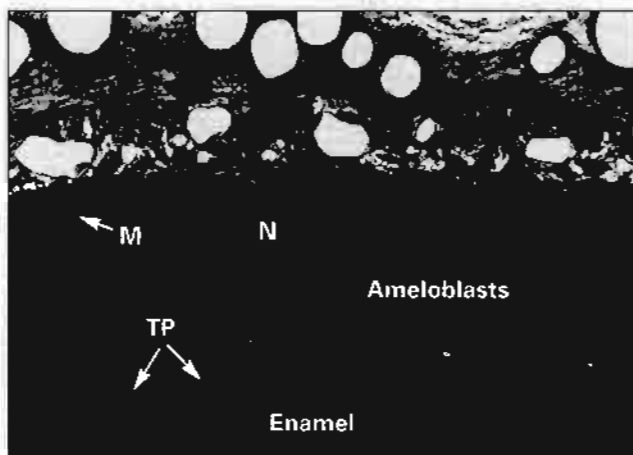


Fig 3-1 Secretory ameloblasts of the rat incisor tooth. In the secretory stage, ameloblasts are tall columnar cells, characterized by a secretory process (Tomes process [TP]) and an infranuclear concentration of mitochondria (M). (BV) Blood vessels; (N) nucleus; (OEE) outer enamel epithelium; (SI) stratum intermedium.



Fig 3-2 Electron micrograph of the proximal end of secretory ameloblasts (AM) containing a high concentration of mitochondria (M). Cells of the stratum intermedium (SI), stellate reticulum (SR), and outer enamel epithelium (OEE) have not yet formed the papillary layer. Blood vessels (endothelium [E]) lie in close apposition to the OEE. (Original magnification $\times 1,600$.)

the SI cells are relatively narrow, and the adjacent cells are held together by many desmosomes. Large gap junctions are formed with adjacent cells of the IEE. The SI cells contain many mitochondria, a characteristic shared with the distal cytoplasm of secretory ameloblasts.

Although the exact function of the cells of the SI is not clear, the fact that they contain a rich complement of mitochondria and stain intensely for alkaline phosphatase and adenosine triphosphatase (ATPase), suggests a possible role in ion and water transport. The presence of extensive gap junctions between the cells of the SI and the ameloblasts indicates that the two cell types act in close concert during amelogenesis.

Recent studies have shown that the regulatory activity of the enamel knot is taken up by the SI during cusp formation.¹ Growth regulatory signals mediated by Sonic Hedgehog (Shh) are activated in the SI in a wavelike manner from the cusp to cervical zone.

The IEE is a layer of columnar preameloblasts abutting the dental papilla. Organ culture studies of amelogenesis indicate that contact with the dental papilla is required for the expression of enamel protein.² Although the precise nature of the early instructive signals that originate from the dental papilla have yet to be identified, the permissive effect of the extracellular matrix of the basement membrane is required for initiating expression of amelogenin.³

The region of highest mitotic activity in the IEE is located near the cervical loop portion of the enamel organ.⁴⁻⁶ Stem cells in the cervical loop divide to give rise to an expanding metaplast clone of preameloblasts.^{7,8} The rates of proliferation and differentiation in the blast-metaplast populations vary among species, among individual teeth, and among different parts of a given tooth.

Secretion of growth factors by the enamel knot and the dental papilla regulate cell proliferation and growth of the IEE.^{6,9} Receptors for epidermal growth factor, platelet-derived growth factor, and fibroblast growth factor have been localized by immunohistochemistry in preameloblasts.¹⁰ Phospholipase C γ , a downstream signaling molecule activated by growth factor-receptor interaction, was also demonstrated in preameloblasts. Preameloblasts must exit the cell cycle to begin the process of cytodifferentiation.⁴

The cytoplasm of the preameloblasts contains many free ribosomes, a small Golgi apparatus, a few cisterns of rough and smooth endoplasmic reticulum, and a small number of mitochondria. Adjacent preameloblasts form gap junctions and desmosomes. A prominent zonula adherens junction with an associated terminal web of cytoplasmic filaments forms in the proximal cytoplasm adjacent to the cells of the SI.^{11,12} This proximal junctional complex binds and stabilizes the preameloblasts prior to degradation of the underlying basal lamina.

Soon after the deposition of mantle dentin, the odontoblasts express matrix metalloproteinases (MMPs) that begin the digestion of the basement membrane. Preameloblasts subsequently remove basement membrane fragments through phagocytosis.¹³ Removal of the basement membrane allows the dentin and enamel matrices and their respective mineral phases to come into direct contact. As a result, a strong mechanical bond is formed between the enamel and dentin.

Recent studies using reverse transcription and polymerase chain reaction techniques have shown that immature enamel organ cells exist in a "protodifferentiated" state.¹⁴ Preameloblasts of the IEE produce small amounts of enamel matrix protein prior to overt morphodifferentiation as secretory phenotypes.^{15,16} Some of these proteins traverse the dental lamina and are taken up in odontoblast-coated vesicles.

Although the signal transduction pathways that regulate ameloblast and odontoblast differentiation have yet to be identified, immunohistochemical evidence has shown that cytokine-activated signaling pathways, including protein kinase C activation, are involved in early amelogenesis.¹⁷

Structure of Secretion-Stage Ameloblasts

In describing the structure of the ameloblast, the term *proximal* is used to refer to the end of the cell nearest to the SI, and the term *distal* is used to identify the secretory end of the cell, next to the enamel (see Fig 3-2). The term *apical* is also used to describe the secretory pole of ameloblasts.

The structural changes that characterize each of the various stages of ameloblastic activity have been well documented.¹⁸⁻²¹ The cytoplasm of the mature secretory ameloblast is highly polarized (Figs 3-1 to 3-4). The infranuclear (proximal) cytoplasm contains many mitochondria and a terminal web of cytoplasmic filaments associated with a zonula adherens (see Fig 3-2). Gap junctions are present between the proximal surface of the ameloblast and the overlying cell of the SI.

The supranuclear (distal) cytoplasm, which accounts for more than one half of the total cell volume, is filled with an extensive array of RER cisternae and a well-developed Golgi complex (see Figs 3-4a to 3-4c).^{18,20,22} Electron microscopic autoradiography and immunocytochemical studies have shown that enamel proteins are synthesized in the RER and glycosylated in the Golgi cisternae prior to being

packaged into specific granules (see Figs 3-4a to 3-4c).²³⁻²⁵ Condensing vacuoles derived from the trans-Golgi network mature into smaller, dense-core secretory granules. The newly formed secretory granules are immediately transported along a microtubular network to the distal end of the cell, where they are released by merocrine secretion into the enamel compartment.^{26,27} Microtubule inhibitors, such as colchicine and vinblastine, block enamel matrix secretion.²⁸ A distal junctional complex, consisting of gap junctions, a zonula adherens, and a zonula occludens, bind adjacent ameloblasts and seal the lateral intercellular spaces from the enamel-forming compartment (see Fig 3-3).²⁹

The first layer of enamel matrix (about 20 μ m) is secreted across the flat distal cell surface of the newly differentiated ameloblast. As new membrane is added to the distal plasma membrane by the fusion of matrix secretion granules, the distal cell surface develops a protruding cytoplasmic process, 5 to 15 μ m in length (see Figs 3-1, 3-3, and 3-4). Sir John Tomes, a British dentist and histologist, first described this process in the mid-19th century. The formation and the length of Tomes process (TP) appear to be related to the quantity and speed of matrix secretion, because new secretory granule membrane is added to the secretory pole of the cell faster than it can be recovered and recycled from that region. Tomes process protrudes at an angle to the long axis of the ameloblast cell body (see Fig 3-1).

With the formation of TP, the secretory surface of the ameloblast becomes more complex, and secretory granules are directed to two regions of the distal cytoplasm.^{12,26,30} Enamel matrix proteins released from the distoventral part of TP form prismatic or rod enamel (see Figs 3-3 and 3-4). Secretion from the proximal part of TP, at the point where adjacent ameloblasts abut each other, gives rise to the interprismatic or interrod enamel (see Fig 3-2). The plasma membrane is highly infolded and apparently continuous with a tubulovesicular compartment at both the proximal and distal secretory sites.^{25,27} The species-specific prism pattern is genetically determined by the shape and hexagonal packing of ameloblasts, the orientation of TP vis-à-vis the cell body, and the rate of enamel deposition.³¹⁻³³

Observation of TP by electron microscopy has led to the conclusion that its surface can be subdivided into a secretory face (the concave or ventral surface) and a retrieval face (the convex or dorsolateral surface) (see Fig 3-3).³⁴ Endocytosis for retrieval of membrane is carried out by formation of coated vesicles along the nonsecretory plasma membrane. Internal-

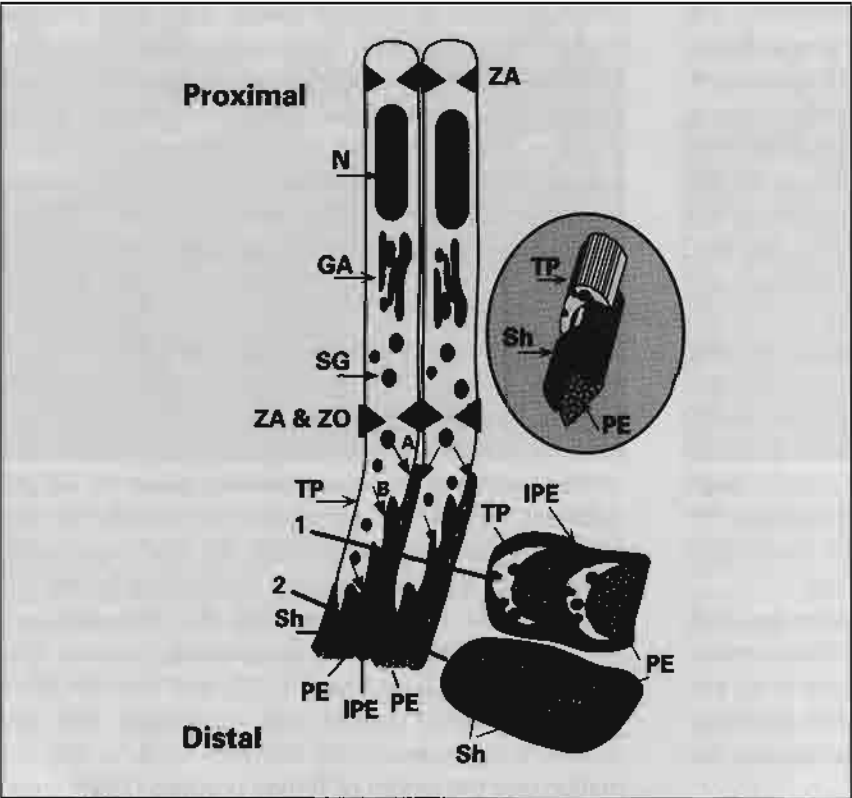
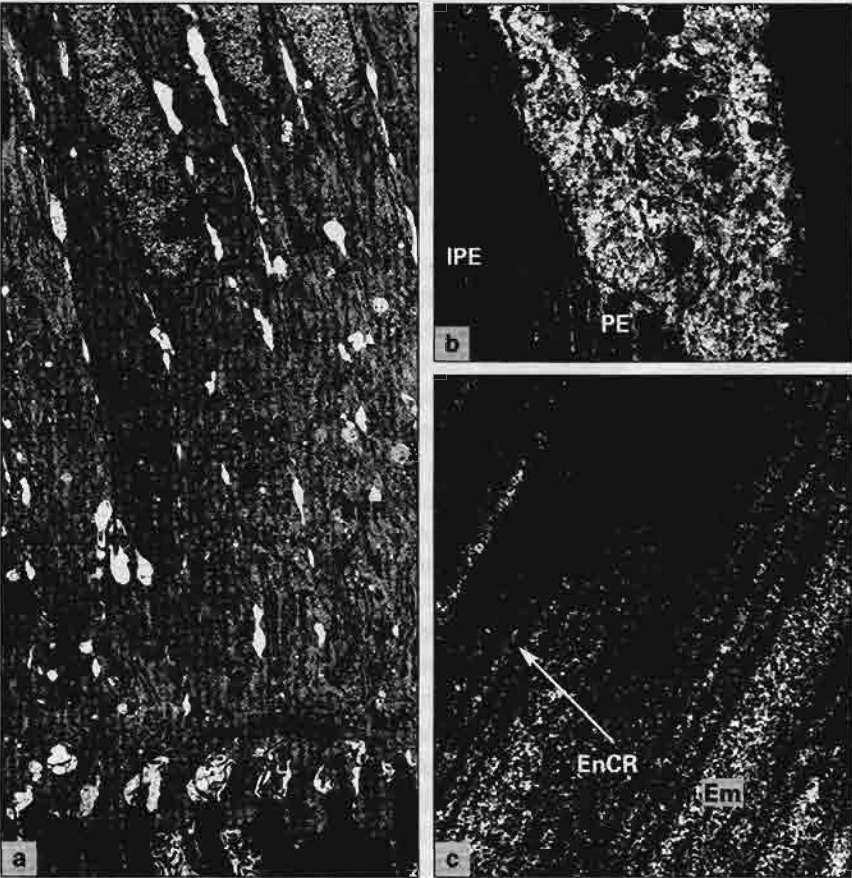


Fig 3-3 Secretory ameloblasts. A zonula adherens (ZA) junction binds adjacent ameloblasts at both proximal and distal ends. The bulk of the infranuclear cytoplasm is occupied by the rough endoplasmic reticulum and a well-developed Golgi apparatus (GA). A zonula occludens (ZO) barrier is present in the intercellular space just proximal to Tomes process (TP). Secretion granules (SG), originating in the GA, are secreted from the proximal end of TP (A), giving rise to interprismatic enamel (IPE). Additional SG discharge at the distal end of the process (B) gives rise to a single enamel prism (PE). Interprismatic enamel is contributed by several contiguous cells. Cross sections of TP (1) and the adjacent enamel (2) illustrate the relationship of the secretory surface to prismatic enamel and the endocytotic surface to the development of a prism sheath (Sh). (N) Nucleus. (inset) Relationship among the prism sheath, the prism, and Tomes process.



Figs 3-4a to 3-4c Electron micrographs of secretory ameloblasts. (a) Distal portion of the ameloblasts, containing Golgi complexes (G) and an abundance of rough endoplasmic reticulum (RER). Secretion occurs from Tomes process (TP). (Nuc) Nucleus; (IPE) interprismatic enamel. (Original magnification $\times 2,200$.) (b) High magnification of Tomes process (TP), containing secretion granules (SG). (IPE) Interprismatic enamel; (PE) prismatic enamel. (Original magnification $\times 17,000$.) (c) Regular spacing of developing enamel crystallites (EnCR) in the enamel matrix (Em). (Original magnification $\times 94,000$.)

ization and subsequent fusion of coated vesicles form endosomes and multivesicular bodies, components of the cell's digestive apparatus. Studies of the fate of injected tracer substances have shown that they are taken up in coated vesicles of TP, suggesting that solutes in the extracellular space, including initial breakdown products of the enamel matrix, might begin to be removed in relatively small amounts during the secretory stage of amelogenesis.

During initial formation of enamel and during the last few days of enamel deposition, ameloblasts have no TP, and thus no prismatic pattern is formed; therefore, the first few microns of enamel next to dentin, and the last several microns of enamel at the surface, are aprismatic. The crystallites of aprismatic enamel are tightly packed and aligned perpendicular to the enamel surface. Aprismatic surface enamel compromises adhesion of dental occlusal sealants and orthodontic brackets by interfering with the penetration of adhesives into the enamel.³⁵ This layer should be removed by acid etching before treatment protocols that require bonding to enamel.

Each ameloblast forms a single prism or enamel rod (see Fig 3-3).^{12,34,36} The enamel prism is made up of thousands of hydroxyapatite crystallites, oriented more or less parallel to each other. Each enamel crystallite is a ribbonlike structure that is believed to extend without interruption from the dentinoenamel junction to the enamel surface.³⁷ Ultrastructural studies of enamel show that individual crystallites follow a spiral course within the prism.²⁶ In longitudinal sections, enamel prisms exhibit optical cross striations, about 3.7 μm apart, caused by slight constrictions in the width of the prisms due to a daily cyclical rhythm of enamel matrix secretion.³⁸ When human enamel is viewed in cross section, the prisms have an arc-shaped outline and are arranged in offset horizontal rows (see Fig 3-3).³¹

Packing irregularities of crystallites demarcate the prismatic and interprismatic domains. This border region retains protein to form a sheathlike structure. Interprismatic crystallites have their long axes oriented at an angle to those in the prism (see Fig 3-3). The distinction between interprismatic and prismatic enamel is believed to reside solely in the orientation of crystallites. There is no evidence to suggest that the biochemical compositions of the interprismatic and prismatic matrices are different. Physicochemical forces, rather than biochemical differences in matrix proteins, act to orient the matrix and determine crystallite orientation at each of the two secretion sites.²⁶

A prism sheath (Figs 3-3 and 3-5) delimits approximately three quarters of the boundary between pris-

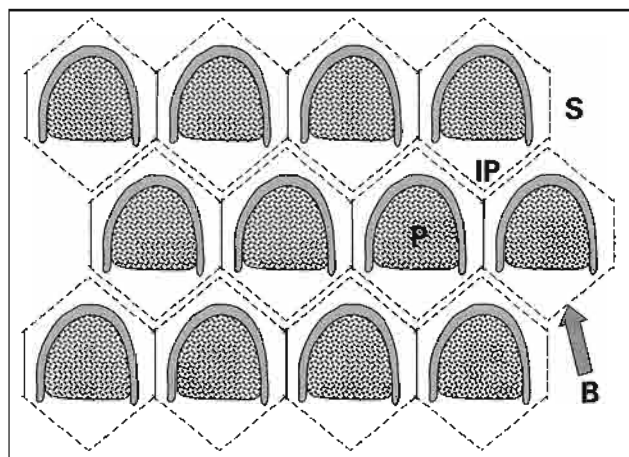


Fig 3-5 Cross-sectional arrangement of the prisms in human enamel. The position of each ameloblast in relation to the prism outline is represented by the superimposed boundary lines (B). Each arcade-shaped prism is surrounded by interprismatic enamel (IP), which is contributed by the secretions of seven ameloblasts. Note the offset arrangement of the horizontal rows of arcades. (P) Prismatic enamel; (S) sheath region.

matic and interprismatic enamel. The composition of the sheath and its manner of development are not well understood. However, the shape of the sheath and its location over the convex surface of the prism suggest that its formation is associated with the endocytotic surface of Tomes process.

No sheath is present, and prismatic enamel is in direct contact with interprismatic enamel, along the flat surface of the prism, corresponding to the secretory surface of TP (see Fig 3-3). During enamel secretion, Tomes process undergoes fragmentation at its most distal point. This may create space between the already mineralized prismatic and aprismatic matrices.³⁹ Several nonamelogenin proteins, collectively known as *sheath proteins*, appear to localize along TP, and in the space along prism boundaries.⁴⁰ The space created along the prism boundary may provide a route for the escape of enamel matrix degradation products during enamel maturation. Retention of enamel protein fragments in the space created by the irregular packing of crystallites at the border between interprismatic enamel and prismatic enamel may contribute to the prism sheath.

Fully matured enamel provides a hard, wear-resistant surface. Its only weakness, relative brittleness or susceptibility to crack formation, is along cleavage planes that follow the border between prismatic and interprismatic enamel.⁴¹ Biomechanical analysis of the fracture behavior of teeth has shown that the dentinoenamel junction undergoes plastic

deformation to help resist crack propagation into the underlying dentin.⁴² Thus, most cracks are confined to enamel. It has been suggested that coarse collagen fibrils in the dentinoenamel junction resist and deflect crack propagation.

Biology of the Enamel Matrix

Information about the composition, mechanism of action in mineralization, and maturational change of enamel matrix proteins has been difficult to obtain because many enamel proteins are present in only relatively small amounts, and most undergo proteolytic processing soon after secretion. However, through the application of molecular biology techniques, significant progress is now being made in this area.⁴³ Current understanding is that ameloblasts produce two classes of matrix proteins: amelogenin, a relatively homogenous product, which constitutes approximately 90% of newly secreted enamel matrix, and a heterogenous group of non-amelogenin proteins, including tuftelin, ameloblastin, enamelin, metalloproteinase, and serine proteinases, which make up the remaining 10%. The role of the enamel epithelium and the enamel matrix proteins in the mineralization of enamel has been the subject of several in-depth reviews.⁴³⁻⁴⁵

Amelogenins

Amelogenins are expressed as several isoforms through alternative splicing of pre-messenger ribonucleic acids (mRNAs).^{12,25,30,46-49} The amelogenins are rich in proline, leucine, glutamic acid, and histidine. Upon secretion the amelogenins form aggregates (Fig 3-6). The hydrophilic carboxy terminals of the amelogenins are exposed at the surface of the aggregates, facing the water-mineral phase. The external anionic surface, containing phosphorylated serine, is believed to play a role in controlling crystal growth.^{50,51} Following the cleavage of the hydrophilic terminals, the amelogenins self-assemble into supramolecular nanospheres approximately 18 nm in diameter (see Fig 3-6).⁴⁹⁻⁵⁴ Each nanosphere comprises 100 to 200 amelogenin molecules stabilized by intermolecular hydrophobic interactions. High-resolution electron microscopy of newly secreted enamel matrix reveals nanospheres aligned between long, ribbonlike crystals of newly formed enamel. Presumably, adjacent crystallites are prevented from lateral fusion by the intervening amelogenin nanospheres, yet are able to grow rapidly along their

C-axis.^{49,53,55} It is postulated that a scaffold of amelogenin nanospheres controls the orientation of the C-axis (long axis) of the developing hydroxyapatite crystals (see Figs 3-4 and 3-6).^{49,53,54,56}

Amelogenins are secreted as 25-kDa molecules that undergo progressive breakdown in the extracellular space. Proteases secreted by the enamel organ carry out specific and sequential proteolytic processing of the amelogenins.⁵⁷⁻⁶² The heavier amelogenins aggregate to form nanospheres that provide a structural scaffold to support the rapid and lengthwise growth of the crystallites (see Fig 3-6).⁶³⁻⁶⁶ The smaller (20- and 13-kDa) fragments may slow crystallite growth in width and thickness by controlling the ionic activity of calcium in the enamel fluid.^{64,66,67}

As the amelogenins complete their function, they are resorbed from the enamel matrix. Proteolytic enzymes from two classes of proteases, the serine proteases and the matrix metalloproteinases, appear responsible for degradation of enamel matrix (for review, see Woessner⁶⁰). Degradation of amelogenin is accomplished by specific proteinases produced by secretory and maturation ameloblasts. Cleavage of the hydrophilic carboxy terminal peptide by a serine protease initiates the degradation process.⁶⁸ The rest of the amelogenin molecule is degraded by another serine proteinase (ameloprotease I), which appears to be a component of the enamelin fraction.⁶⁹

Recent studies suggest that metalloproteinases are involved in matrix degradation during the secretory-to-transition phase and that serine proteinases function mainly during the maturation phase.⁴³ In situ hybridization and immunohistochemistry indicate that MMP-20 (enamelysin) is expressed in secretory ameloblasts and odontoblasts.⁷⁰ Secretory ameloblasts may, to a limited extent, remove amelogenin peptides by endocytosis along the dorsal surfaces of Tomes process. However, the bulk of the amelogenin breakdown products are removed by maturation ameloblasts following the completion of the full thickness of the enamel layer.

Nonamelogenins

The nonamelogenin protein fraction contains relatively large (28- to 130-kDa) proteins of a generally acidic and hydrophilic nature. Several specific gene products have now been identified in the nonamelogenin fraction. These include tuftelin, ameloblastin, enamelin, and proteinases. Proteins of the nonamelogenin fraction demonstrate high binding affinity for hydroxyapatite crystals.⁷¹ These proteins are retained in small quantities in fully matured enamel in the

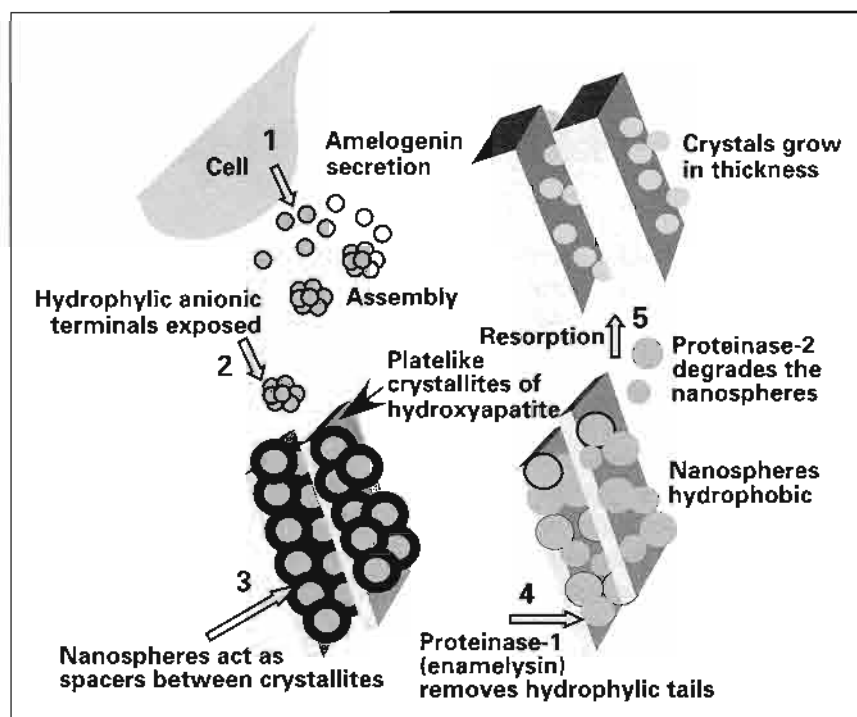


Fig 3-6 Current concept of the role of amelogenins in the mineralization of enamel. The hydrophobic amelogenins form globular aggregates (nanospheres) on secretion into the extracellular space. The nanospheres form lattices that regulate the spacing and the orientation of the C-axis of the newly forming enamel crystallites. (Adapted from Fincham et al⁵¹ with permission from Elsevier Science.)

prism sheaths and as thin coatings surrounding the crystallites.

Tuftelin is a specific nonamelogenin acidic protein found in high concentration near the dentinoenamel junction and within enamel tufts.^{21,72} Enamel tufts are hypomineralized developmental defects that extend perpendicularly from the dentinoenamel junction into the enamel. Tuftelin is the first enamel protein to be expressed during IEE differentiation. Tuftelin is a glycosylated protein with serine and threonine phosphorylation sites. Because of its composition, its early secretion, and its concentration at the mineralization front, tuftelin could have a role in nucleation of enamel crystallites.⁷³ The human tuftelin gene has been localized on chromosome 1.⁷⁴

Ameloblastin, amelin, and sheathlin form a group of related "sheath" proteins that have been detected in rat, human, and porcine enamel.^{16,40,75-78} Ameloblastin and sheathlin proteins accumulate between the plasma membrane of Tomes process and the growth zone of enamel prisms.^{16,79} It has been suggested that sheath proteins may serve an early adhesive function in stabilizing the nonsecretory surface of TP to the enamel matrix.^{77,80}

Soon after their secretion, the parent molecules undergo cleavage. The C-terminal polypeptide frag-

ments are rapidly degraded and removed from the enamel. However, the N-terminal ameloblastin and sheathlin polypeptides are retained in prism sheaths.^{79,80} Ameloblastin degradation products are less soluble than the parent molecule.⁴³ Nonamelogenin protein fragments are believed to account for most of the remaining small percentage of protein contained in fully mature enamel.

Of additional interest is the report that amelin mRNA has been localized in preodontoblasts before it is expressed in ameloblasts.⁷⁸ It has been suggested that amelin may have a role in the presecretory epithelial-mesenchymal interaction.

Enamelin is a high-molecular weight, acidic, glycosylated protein. It is secreted as a 186-kDa entity that subsequently undergoes progressive degradation to a 32-kDa molecule.⁸¹ The later fragment binds readily to enamel crystallites.⁸² The higher molecular weight fractions are localized along Tomes process and the newly developing enamel prism. The smaller fractions are located more deeply in the enamel, in association with the mineral in the prismatic and interprismatic domains.^{81,83}

Some acidic proteins of the enamel fraction are now known to be precursors of a group of serine proteinases that degrade enamel matrix.^{58,68,69,84}

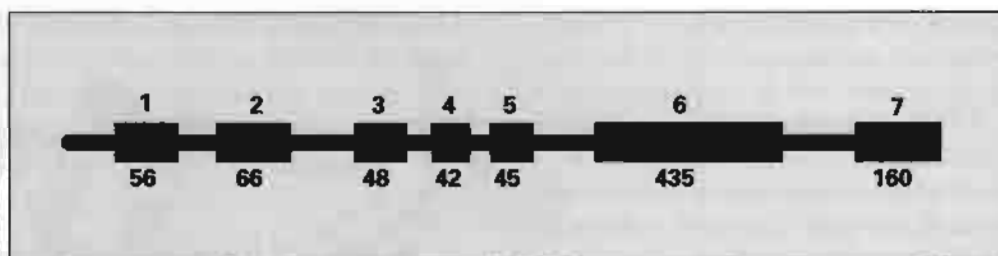


Fig 3-7 Structure of the X-chromosomal copy of the human amelogenin gene. The bar segments represent the introns and the boxes (1 through 7) correspond to the exons. The nucleotide numbers are indicated below the exons. (Adapted from Simmer et al.¹⁷³)

Despite recent progress in the biochemical characterization of the enamel matrix, knowledge of the sequential biochemical and biophysical interactions between mineral ions and enamel matrix proteins is still very incomplete. The most recently postulated roles for amelogenin and nonamelogenin proteins in initiating and controlling the construction of enamel have been reviewed by Nanci et al,¹⁶ Robinson et al,⁴³ and Fincham et al.⁵¹

Location and Expression of Amelogenin, Ameloblastin, and Tuftelin Genes

The gene for amelogenin (*AMEL*) has been mapped to the sex chromosomes (Fig 3-7). In the rat, hamster, and mouse, *Amel* is present on the X chromosome⁸⁵; in humans, *AMEL* is present on both the X and Y chromosomes.^{86,87} The gene on the Y chromosome (*AMELY*) is located in the q11 region, and the *AMELX* gene is located on the distal short arm (p22.1 to p22.3 positions) of the X chromosome. Recombination errors during the duplication of the sex chromosomes can lead to amelogenesis imperfecta.

The human amelogenin gene has seven introns and seven exons (see Fig 3-7). Both the X and Y amelogenin gene copies are expressed during tooth development. Transcription of the *AMELX* message appears several times more active than that of the Y copy, and the level of X-chromosomal amelogenin mRNA has been measured to be several fold higher than that of Y-chromosomal amelogenin mRNA.

A variety of amelogenin proteins are produced by alternative splicing of pre-mRNA.⁴⁶⁻⁴⁸ Exons, and

parts of exons, are deleted during alternative splicing. The resulting proteins all have a hydrophobic amino terminal, a large hydrophilic middle polypeptide, and a hydrophilic carboxy terminal. It is unclear if each amelogenin isoform performs a different function during enamel formation.

A small deletion in the *AMELY* gene permits it to be distinguished from its *AMELX* counterpart. This difference has proven useful in sex identification of human remains recovered from archeological sites⁸⁸ and in forensic science.⁸⁹

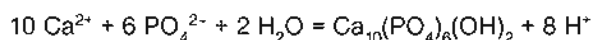
The human tuftelin gene is located on chromosome 1.^{74,90} The ameloblastin gene is localized to chromosome 4q21.⁹¹

Mineralization of the Enamel Matrix

At the onset of enamel formation, the first enamel crystallites are spatially separated from the smaller dentin crystallites. High-resolution electron microscopy of the dentinoenamel junction indicates that the earliest enamel crystallites form from the alignment of dotlike mineral nuclei, approximately 2 to 4 nm in diameter.⁹² Chainlike association of these nuclei, apparently controlled by the amelogenin organic matrix, gives rise to long, needle-shaped crystallites. The crystallites develop in small clusters within extracellular deposits of amelogenin matrix, having the appearance of stippled material in electron micrographs.

Biochemical and electron probe analysis of the earliest crystallites suggests that the first mineral phase to be formed is a two-dimensional octacalciumphosphate precursor that subsequently transforms into hydroxyapatite.^{66,93} The smallest hydroxy-

apatite crystal units (unit cells) are formed by the following reaction:



The hydrogen ions generated during crystal formation must be buffered to maintain a neutral pH to allow continued matrix mineralization.⁴⁴

Enamel crystal growth occurs in a compartment isolated between mineralized dentin and the zonula occludens junction of the ameloblastic layer. Elemental analysis indicates that the fluid in the mineralization compartment has a different composition than serum and extracellular fluid.^{94,95} The presence of a distal zonula occludens junction between ameloblasts and the histochemical demonstration of calcium ATPase activity in the plasma membrane of Tomes process suggest that ameloblasts might control the fluid milieu within which enamel is deposited.⁹⁶⁻⁹⁸

Calcium ATPase has also been demonstrated in the distal cytoplasm of maturation ameloblasts.⁹⁸ The recent localization of Ca^{2+} pump proteins in human secretory and early-stage maturation ameloblasts provides additional support for a functional plasma membrane calcium pump.⁹⁹ The highest concentration of calcium pump protein was localized in the distal ends of ameloblasts near the mineralized enamel.⁹⁹

It has been proposed that the intracellular transport of calcium could be carried out by several calcium-binding proteins localized in both secretory and maturation ameloblasts.¹⁰⁰⁻¹⁰² The recent identification of two low-affinity, high-capacity, calcium-binding proteins (calreticulin and endoplasmic reticulum) in the endoplasmic reticulum of secretory and maturation ameloblasts provides support for a new theory of calcium transcytosis involving the endoplasmic reticulum and inositol triphosphate-gated calcium channels.¹⁰³ The endoplasmic reticulum could serve as a high-volume conduit for calcium transport across ameloblasts without altering the normal cytosolic calcium concentration. This theory would also explain why a large amount of endoplasmic reticulum but low levels of secretory protein synthesis are found in maturation ameloblasts.^{103,104}

In situ hybridization with complementary deoxyribonucleic acid (cDNA) probes for bone sialoprotein is strongly positive in secretory ameloblasts. The potential role of bone sialoprotein, a calcium-binding protein common to most mineralized tissues, in enamel mineralization remains to be determined.¹⁰⁵ It has been suggested that tuftelin and/or bone

sialoprotein could trigger enamel crystal nucleation.^{43,73}

Structure of Transition-Stage Ameloblasts

On completion of the full thickness of enamel, the secretory ameloblasts undergo cytoplasmic reorganization as they switch from a primarily protein secretory cell to that of an absorptive and transport cell. This process is characterized by extensive intracellular digestion of parts of the RER and other cytoplasmic organelles inside autophagosomes. During this stage, the ameloblasts contain high levels of acid phosphatase, indicative of increased lysosomal enzyme activity. The transition stage remodeling is so intensive that approximately 25% of the ameloblasts undergo programmed cell death.^{106,107}

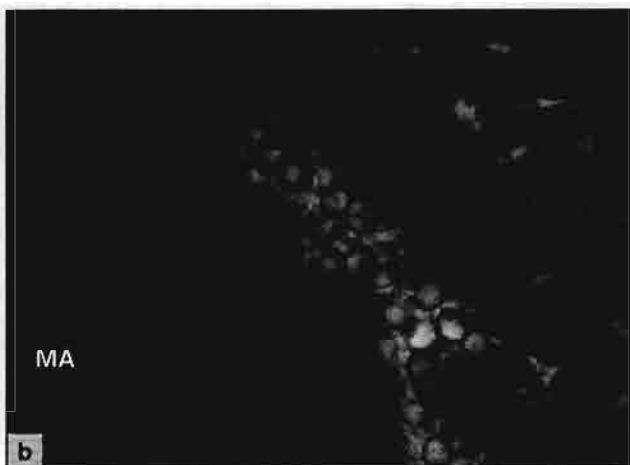
Surviving cells contain less RER, and their Golgi complexes contain many smooth vesicles and lysosomal-like structures. The development of a ruffled border, against the surface of the mineralized enamel, signifies the start of the rapid removal of water and protein from the enamel. At the completion of transition, the ameloblasts are shortened to half their previous height (Figs 3-8a and 3-8b). They are now referred to as *maturation ameloblasts*.

Formation of the Papillary Layer

During the final phase of secretion, and progressing through the transition stage, the OEE, SR, and SI are transformed into the papillary layer, an epithelium believed to be specialized for transport.¹¹ This conversion is preceded by a reduction in the size of the intercellular spaces of the SR and by a loss of glycosaminoglycans. The redifferentiated cells of the OEE, SR, and SI arrange themselves into numerous epithelial folds, or papillae, located between the ameloblasts and a well-developed capillary bed (see Figs 3-8a and 3-8b).

The former OEE, SR, and SI cells are no longer distinguishable as separate cell types, and are now referred to as *papillary cells*. Papillary cells contain numerous mitochondria, large numbers of pinocytotic vesicles, and extensive gap junctions.¹⁰⁸⁻¹¹¹ Numerous microvilli increase the papillary cell surface area several fold.

The cytoplasmic features of the papillary cells, along with their association with a rich bed of fenestrated capillaries, suggest that at this stage the



Figs 3-8a and 3-8b Papillary layer (PL) cells situated between the capillaries and the maturation ameloblasts (MA). (Hematoxylin-eosin stain. Original magnification $\times 600$.) (a) Cross section depicting the MA through their long axis, and the alternating arrangement of papillae and indenting blood vessels. (E) Endothelial cells. (b) Tangential section through the maturation enamel organ, illustrating the close contact between papillary cells and capillaries filled with red blood cells (rbc).

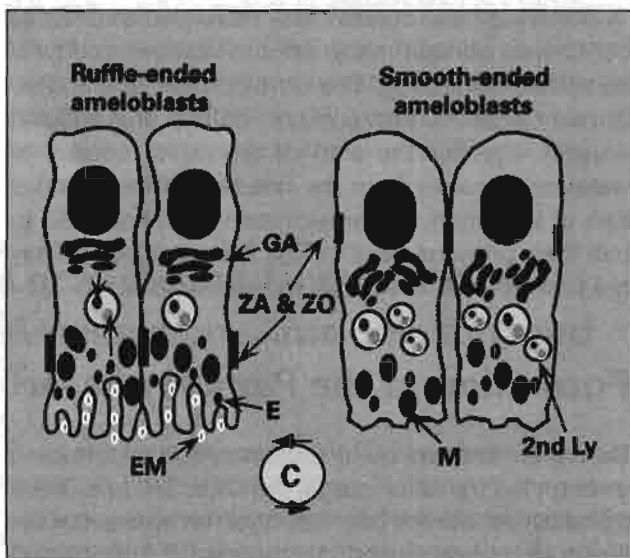


Fig 3-9 Maturation ameloblast phenotypes. Ruffle-ended and smooth-ended maturation ameloblasts cycle back and forth during the maturation phase. Cycling (C) of the two phenotypes involves extensive remodeling of the distal cytoplasm and junctional complexes at both ends of the cells. The Golgi complexes (GA) and the lysosomal (Ly) apparatus are well developed in both cell configurations. Zonula adherens (ZA) and zonula occludens (ZO) shift from a distal position in the ruffle-ended ameloblasts to a proximal position in the smooth-ended ameloblasts. Mitochondria (M) are located primarily in the distal cytoplasm. Endosomes (E) containing enamel matrix (EM) are present in highest amount in the ruffle-ended ameloblasts.

enamel organ has become specialized to perform transport functions related to enamel maturation.¹¹² The fact that papillary cells form extensive gap junctions with adjacent maturation ameloblasts leads to the conclusion that these two types of cells are acting in concert during maturation.¹¹

Papillary cells have been shown to endocytose exogenous tracer material and to transport it to lysosomal bodies.¹¹¹ This has led to the speculation that the papillary layer participates directly in the removal and degradation of enamel matrix breakdown products that gain access to the intercellular spaces of the pap-

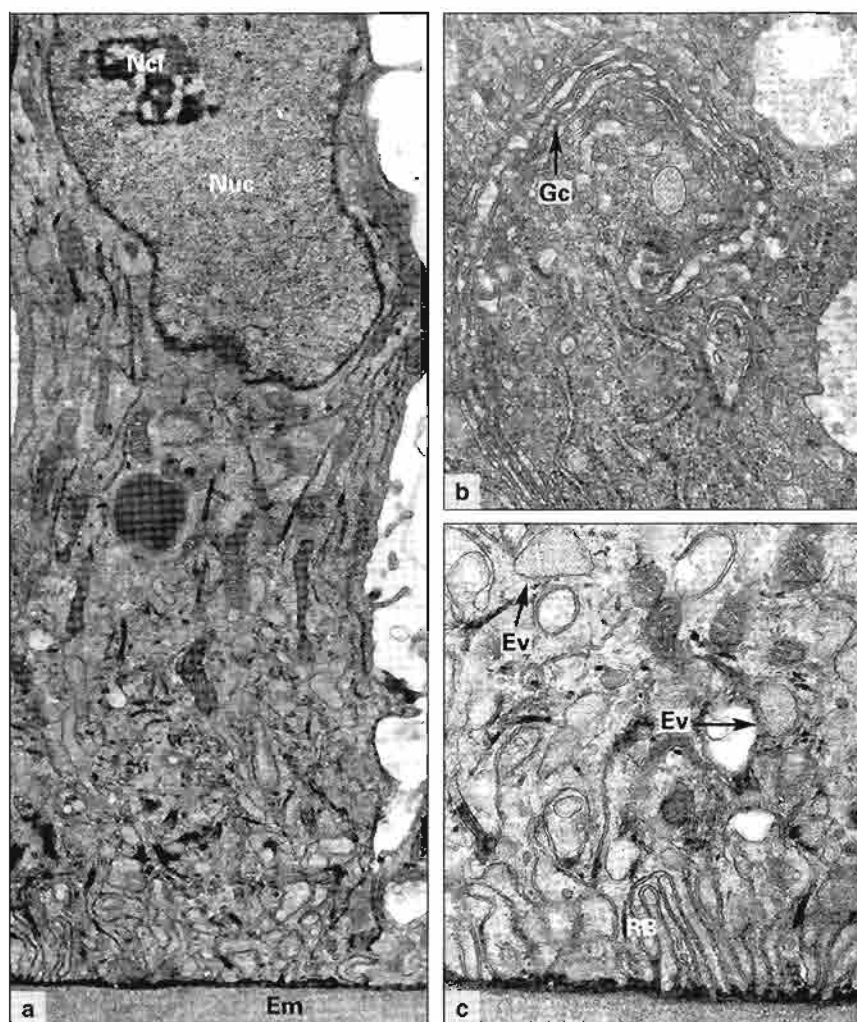
illary layer. However, there is no direct evidence that enamel matrix degradation products diffuse into the intercellular spaces of the papillary layer.

An alternative hypothesis suggests that sodium-potassium-ATPase activity in papillary cells generates an intercellular osmotic gradient across the enamel, drawing water and small matrix polypeptides toward the maturation ameloblasts.¹¹³ The polypeptide matrix fragments would undergo endocytosis and additional degradation in secondary lysosomes of maturation ameloblasts (Figs 3-9 to 3-11).

Fig 3-10a Distal part of a ruffle-ended maturation ameloblast. (Em) Enamel matrix; (Ncl) nucleolus; (Nuc) nucleus. (Original magnification $\times 9,000$.)

Fig 3-10b Golgi complex containing numerous Golgi cisternae (Gc) in a maturation ameloblast. (Original magnification $\times 16,000$.)

Fig 3-10c Ruffled border (RB) and endocytosis vesicles (Ev) at higher magnification. (Original magnification $\times 20,000$.)



Structure of Maturation-Stage Ameloblasts

During the maturation stage, water and enamel matrix degradation products are removed from the enamel, and mineralization continues until the final enamel achieves a composition (by weight) of 95% mineral and only 4% water and 1% organic matrix.^{26,114} Biochemical analysis of enamel indicates that there is a rapid loss of matrix during the initial phase of maturation. Prior to this stage, the enamel is soft and porous, and the crystallites have yet to grow to their final thickness.¹¹⁵ During the final stages of the maturation process, water is lost as mineral continues to be added to the growing crystallites. Ever-smaller quantities of matrix proteins are released and removed by the maturation ameloblasts until the enamel reaches its mature state prior to eruption.

Maturation ameloblasts (and perhaps the secretory ameloblasts) contribute proteolytic enzymes that are involved in an extracellular enzymatic cleavage of matrix proteins into small peptides prior to removal by endocytosis.^{84,116,117} One such enzyme is enamelysin, a matrix metalloproteinase (MMP-20) that degrades amelogenin.⁵⁹ A serine proteinase (ameloprotease) capable of degrading the entire amelogenin molecule has been isolated from pig enamel matrix.^{69,118} Membrane-type matrix metalloproteinase (MT-MMP) is also expressed in ameloblasts.¹¹⁹ It has been suggested that MT-MMP might function as an activator of extracellular MMPs close to the cell surface during enamel maturation.

Enamel maturation is more time consuming than the preceding secretory stage. Maturation ameloblasts remain in contact with the enamel surface for approximately two thirds more time than do the secretory ameloblasts. Failure of enamel maturation

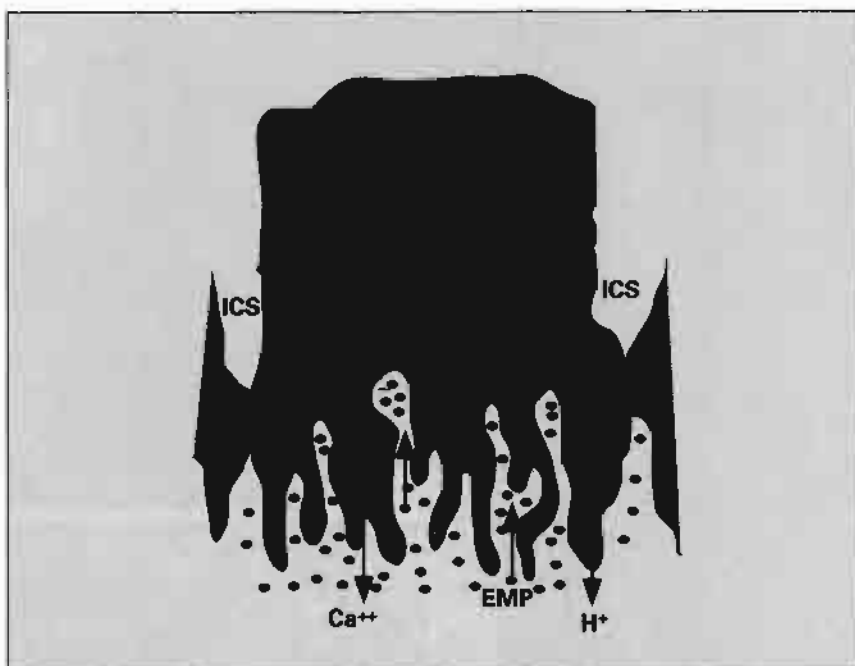


Fig 3-11 Proposed pathway of enamel protein (EMP) reabsorption and digestion by ruffle-ended ameloblasts. The intercellular space (ICS) is sealed by a zonula occludens (ZO). Enamel proteins are endocytosed from the labyrinthine spaces of the ruffled border into endosomes (E) that fuse with larger secondary lysosomes (SL). Lysosomal enzymes are transported to the SLs via primary lysosomal vesicles (L) originating in the area of the Golgi apparatus. H⁺-adenosine triphosphatase, expressed at high levels, is responsible for secretion of H⁺ into the enamel. High levels of alkaline phosphatase are correlated to calcium transport at the ruffled border. (CV) Coated vesicle.

leads to the eruption of enamel that is relatively soft, porous, and easily discolored by food and/or blood and serum.

On completion of the transition phase, maturation ameloblasts develop a ruffled border, a zone of cytoplasmic folds and invaginations along the distal end of the cell in contact with the enamel (see Figs 3-9 and 3-10).¹²⁰⁻¹²² Freeze-fracture studies of maturation ameloblasts have revealed a high concentration of intramembrane particles, indicative of possible transport and/or receptor-ligand activity at the distal surface.¹²³ Maturation ameloblasts cycle between distal ruffle-ended and smooth-ended morphotypes (see Fig 3-9).¹²⁴⁻¹²⁶

Maturation ameloblasts have well-developed Golgi complexes that contain many lysosomal vesicles. Morphologic, tracer, and autoradiographic evidence suggests that resorption of the enamel matrix occurs from the zone of the ruffled border.^{29,127,128} Unlike secretory ameloblasts, the maturation ameloblasts produce a basal lamina over the surface of the maturing enamel.¹²⁹ Matrix degradation fragments must traverse the basal lamina prior to undergoing endocytosis at the ruffled border. Endocytosis of granular material within vesicles formed in the invaginations of the distal cytoplasm has been observed in all species that have been studied at the electron microscopic level. Additional high-resolution immunocytochemical studies have shown that endocytotic vesicles and secondary lysosomes con-

tain material that reacts with antibodies raised against amelogenin proteins.¹²⁸

It is not known if all amelogenin peptide fragments are removed via the cytoplasmic route or whether some of the small peptides simply diffuse out of the enamel and the enamel organ without passing through the maturation ameloblasts.¹³⁰ The presence of distal zonula occludens junctions between adjacent ruffle-ended maturation ameloblasts suggests that a direct intercellular diffusion pathway is blocked to the free flow of substances from the enamel, at least beneath the ruffled border (see Fig 3-9).^{111,131} The absence of a proximal zonula occludens between ruffle-ended maturation ameloblasts and the absence of a distal zonula occludens between the smooth-ended maturation ameloblasts, however, permit diffusion of peptides from enamel into the intercellular spaces between the smooth-ended maturation ameloblast (see Fig 3-9). From there, degradation products could gain access to the intercellular spaces of the papillary layer by lateral movement through the spaces between the ruffle-ended maturation ameloblasts (see Fig 3-9). This indirect pathway between maturation-phase enamel and the papillary layer and its blood vessels has been demonstrated by the diffusion of tracers.²⁶

A similarity between the activity of ruffle-ended maturation ameloblasts and osteoclastic resorption of bone matrix has been noted. Mannose-6-phosphate receptors for lysosomal enzymes are present on the

ruffle-ended membranes of both cell types, suggesting that the ruffled border of the ameloblast is a target for outward transport of lysosomal enzymes.¹³² Positive immunocytochemical reactivity for cathepsin B in the distal ends of ruffle-ended ameloblasts confirms lysosomal enzyme transport to that location.⁸⁹

In addition to resorption of matrix, the ruffle-ended ameloblasts engage in the transport of calcium into the maturing enamel (see Fig 3-11).¹³³ With the onset of maturation, there is a relatively sharp drop in matrix protein followed by an increase in the rate of mineral incorporation into the enamel. Peaks of mineral acquisition are associated with the presence of the ruffle-ended ameloblasts.^{132,134,135} High levels of calcium-ATPase activity in the ruffle-ended membranes of ruffle-ended ameloblasts appear related to the transport of calcium.⁹⁸

Alkaline phosphatase activity is high in the ruffled border of maturation ameloblasts.¹³⁶ It has been suggested that alkaline phosphatase may generate PO_4 , required during formation of hydroxyapatite. Smooth-ended ameloblasts, in contrast to ruffle-ended ameloblasts, occupy less surface area on the tooth surface, exhibit less intense alkaline phosphatase and calcium-ATPase activity, and are not correlated to areas of calcium incorporation.

Histochemical and immunocytochemical studies have also shown that the ruffle-ended maturation ameloblasts contain proton pumps (H^+ -ATPase) and carbonic anhydrase.^{137,138} It has been proposed that protons generated by carbonic anhydrase activity are transported into the enamel across the membranes of the ruffled border by H^+ -ATPase (see Fig 3-11). The resulting decrease in pH beneath the ruffle-ended ameloblasts might activate proteolytic enzymes required for the degradation of matrix proteins. The large concentration of mitochondria adjacent to the ruffled border could supply the adenosine triphosphate (ATP) for the energy needs of proton transport.

Paradoxically, the role of carbonic anhydrase may also be to generate bicarbonate needed to scavenge hydrogen ions generated during hydroxyapatite formation. Bicarbonate ions could also be supplied from plasma circulating through the fenestrated capillaries. Carbonic anhydrase is also found in early enamel matrix.¹³⁹ Its potential role in mineralization has yet to be clarified.

Maturation ameloblasts do not remain in the ruffle-ended configuration for the duration of the maturation process. The ruffled border is transformed into a smooth distal surface abutting the enamel. This change is accompanied by the loss of the distal zonula occludens (see Fig 3-9). Maturation amelo-

blasts with a flat distal cytoplasmic configuration are called *smooth-ended ameloblasts*. Many lysosomal vesicles and a high acid-phosphatase activity characterize the smooth-ended ameloblasts. The precise role of the smooth-ended ameloblasts in enamel maturation is unknown. They appear to participate in protein degradation.

Entire clones or cohorts of maturation ameloblast undergo cyclic change from the ruffle-ended to the smooth-ended phenotype during maturation (see Fig 3-9).¹²⁵ In the continuously developing incisor of the rat, a total of 45 modulation cycles between the ruffle-ended ameloblast and smooth-ended ameloblast modes have been measured during the maturation of enamel; a mean of 2.8 modulations occurred each day of the 16-day maturation phase.¹²⁶ In teeth that develop more slowly, as in porcine incisors, the modulations occur less rapidly and there are fewer cycles. It remains clear, however, that ruffle-ended ameloblast and smooth-ended ameloblast cycling occurs during the development of all teeth, including those of primates.

Following tooth eruption, interaction of the enamel surface with ions in the oral fluids leads to a small but significant increase in enamel maturation. This post-eruptive maturation, especially if fluoride ion is present in the oral fluids, leads to additional improvement in the surface resistance of enamel to subsequent acid demineralization.

Structure of Postmaturation-Stage Ameloblasts

On completion of maturation, the maturation ameloblasts and the cells of the papillary layer undergo regression, reducing the quantity of their cytoplasmic organelles and their overall size. The postmaturation ameloblasts appear as low columnar cells, and the senescent papillary layer is reduced to one or two layers of low cuboidal cells. The reduced enamel epithelium remains in position, covering and protecting the enamel surface until the erupting tooth makes contact with the oral mucosa. At that stage, the reduced enamel epithelium fuses with the oral epithelium to form the primary junctional epithelium attachment to the cervical aspect of the crown.

Basic Science Correlations

Cytoplasmic organelles undergo rapid change during the many phases in the life cycle of the ameloblasts.

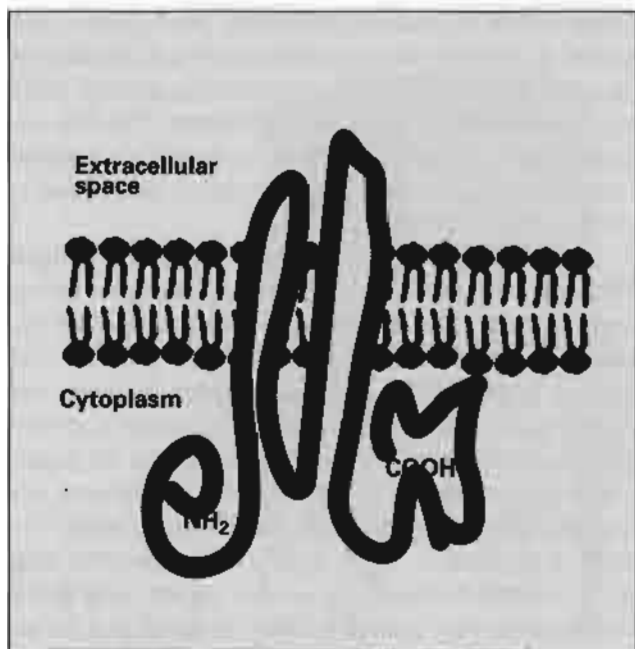


Fig 3-12 Gap junction connexin protein from mammalian liver cells. The amino and carboxy terminals are located on the cytoplasmic surface. Two polypeptide loops of protein extend across the membrane to the external surface of the connexon.

These changes reflect the principal cellular functions that occur at each stage of amelogenesis. For example, the RER is most highly developed in secretory ameloblasts, lysosomes appear most abundant during the transition stage, and endocytotic coated vesicles are unusually prominent in papillary cells during maturation of enamel.

Significant changes in cell-to-cell contacts also occur throughout all phases of enamel formation. They appear to be needed for the coordination of cellular activity and for controlling the compartmentation of the extracellular space. These requirements are met by the formation of gap junctions and zonula occludens junctions. The following sections provide brief reviews of the structure and function of these two junctions.

Gap junctions

Gap junctions provide hydrophilic passageways across adjacent cell membranes for the intercellular exchange of ions and small molecules (less than 1,000 Da).^{140,141} Special transmembrane gap junction

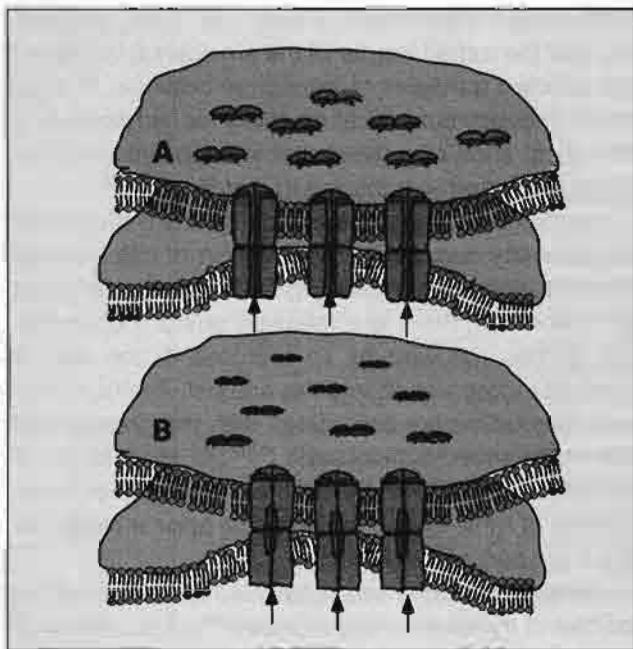


Fig 3-13 Gap junction in the (A) coupled and (B) uncoupled states, showing the association of connexons in two juxtaposed plasma membranes. In the open condition (A), ions and small molecules can move through a fluid-filled pore (green arrows) from cell to cell. When the gap junction is uncoupled (B), the connexons are constricted and the pore is closed (red arrows). (Adapted from Peracchia¹⁴³ with permission from Kluwer Academic.)

proteins, called *connexins* (Fig 3-12), create the channel through the membrane. The connexin molecule has four transmembrane domains, two rather rigid extracellular domains, and two cytoplasmic domains.¹⁴² The carboxy terminal domain, larger than the amino terminal domain, contains amino acid sequences that regulate channel permeability.

Six connexin molecules aggregate in the membrane to form a supramolecular hemichannel, the connexon (Fig 3-13).¹⁴³ In forming a connexon hemichannel, the gap junction proteins assemble with their hydrophobic surfaces facing the lipid phase of the plasma membrane and their hydrophilic surfaces oriented inward (toward each other) to delimit a central fluid-filled channel across the membrane. When connexons from two adjacent cells are connected across the narrow intercellular gap, and the connexons are open, an intercytoplasmic exchange of ions and small molecules may occur. Typical gap junctions are made up of a hundred or more connexons aggregated in complementary patches in the cell membranes of a pair of participating cells (Figs 3-13 and 3-14).

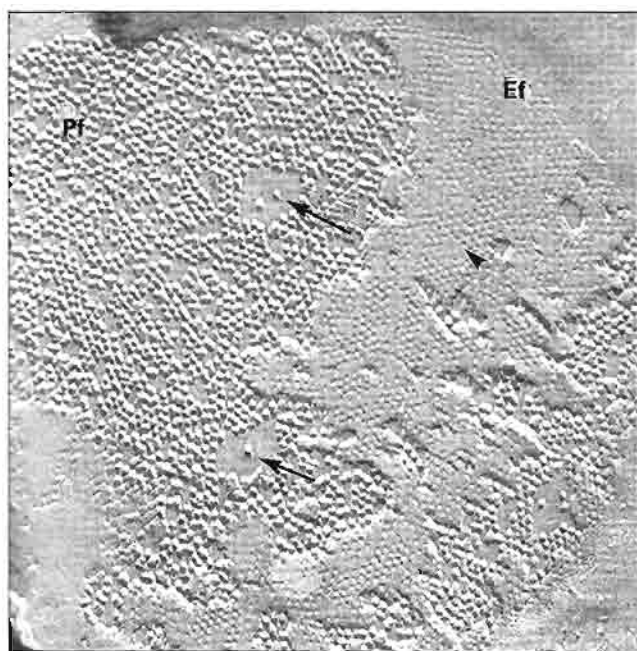


Fig 3-14 Gap junction particles (arrows) are aggregated on the protoplasmic face (Pf) of the fractured plasma membrane. Pits (arrowhead) on the external face (Ef) of the membrane represent the position of the pore of the connexon particle. (Original magnification $\times 92,000$.)

The flow of ions and small metabolites across gap junctions has been shown to be involved in coordinating and regulating cellular activity in groups of contiguous cells. For example, second messengers, such as cyclic adenosine monophosphate, calcium ions, and inositol triphosphate, have been shown to spread through gap junctions.¹⁴⁴ In cardiac muscle, gap junctions, functioning as electrotonic synapses, coordinate the contraction of the heart. Gap junctions are needed during embryonic development to coordinate sequential differentiation of groups of cells.

Gap junction proteins are to some degree tissue specific. Lens, heart, and liver gap junction proteins have different molecular weights, suggesting that their respective connexons have tissue-specific physiologic functions in addition to common properties. The family of genes that encode connexins is made up of at least 12 members.^{141,145} Homotypic and heterotypic assembly of connexin proteins result in gap junctions with different physiologic properties.¹⁴⁶ In addition, it is possible for a single cell type to form different types of connexons and to restrict each type to specific domains of the cell membrane.¹⁴⁷

Participating cells are coupled when adjacent gap junction connexons are open. Various substances regulate the size of the pore opening and thereby control the degree of cell-to-cell coupling (reviewed by Bruzzone et al¹⁴⁸). Cytosolic calcium, cellular pH, retinoic acid, and intracellular oxygen tension have been shown to influence coupling. Connexons close within minutes in response to increased intracellular calcium, acidification of the cytoplasm, and low intracellular oxygen tension. This decoupling represents an emergency shutdown mechanism to prevent a cell-to-cell spread of noxious stimuli.

The calcium-binding protein, calmodulin, has been shown to participate in regulating the action of calcium on connexon proteins. Cyclic adenosine monophosphate modulates the number of gap junctions by increasing the rate of connexon assembly. Since gap junction proteins have a half-life of about 6 hours, there is a constant turnover of connexons at the cell surface.

Gap junctions are present between all cells of the enamel organ, suggesting that intercellular communication is necessary during all phases of enamel development.^{11,12,96,149,150} Immunocytochemical studies have shown that connexin 43 localizes in the SI, IEE, and preameloblasts.¹⁵¹ Information transferred across gap junctions may control cell proliferation and coordinate the activation and subsequent regulation of protein matrix secretion.

Large gap junctions are formed during enamel maturation. This may indicate that a bidirectional flow of ions from ameloblasts to papillary cells is a significant component of cellular activity during enamel maturation. Annular gap junctions are especially conspicuous in papillary cells.¹⁵⁰ The latter are believed to represent stages in the internalization and breakdown of gap junctions.

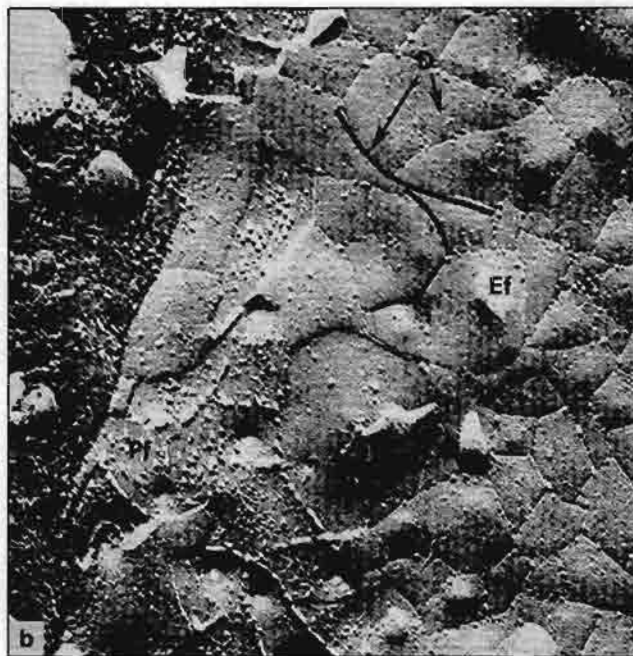
Gap junction proteins have a rapid turnover time of approximately 5 hours. The functional significance of an apparent high turnover of gap junctions during the maturation phase remains to be explored.

Tight junctional complexes

Epithelial cells that are closely juxtaposed may participate in forming zones of fusion (tight junctions) between adjacent plasma membranes. For tight junctions to form, specific proteins must migrate from cytoplasmic pools to the cell surface to be inserted into the plasma membrane at points of cell-to-cell contact. Tight junctional contacts occur either as spotlike macula occludens, larger sheetlike fascia occludens, or as beltlike zonula occludens specializations.



Figs 3-15a and 3-15b Freeze-fracture replica of the distal plasma membrane of a ruffle-ended maturation ameloblast (MAB). (a) Low magnification reveals the ruffled border (RB), components of the zonula occludens (ZO), and a gap junction (GJ). (Original magnification $\times 17,000$.) (b) Higher magnification of the protoplasmic face (Pt). The tight junctional strands (S) of the zonula occludens are visible, as are depressions (D) created by the strands in the external face (Ef). (Original magnification $\times 80,000$.)



The exact significance of the macula and fascia occludens junctions is unclear. Although these junctions cannot compartmentalize an extracellular space, they might provide increased cell-to-cell adhesion, or they might act as intramembrane stabilizers to restrict the lateral diffusion of other integral membrane proteins. In contrast, because the zonula occludens seals the extracellular space in a beltlike zone around the entire circumference of the cell, it compartmentalizes the extracellular space.

The zonula occludens plays two important functions in the physiology of epithelial layers. It provides a variable permeability barrier in the intercellular space, thereby creating isolated compartments and delineating luminal spaces. Second, by preventing lateral diffusion of integral proteins in the plasma membrane, it maintains specific domains in the cell membrane, such as the basolateral and apical surfaces of polarized cells.¹⁵²

The transmembrane protein responsible for creating the seal is called *occludin*. It binds additional proteins, zonula occludens 1 and zonula occludens 2, on its cytoplasmic domain. The zonula occludens proteins are kinases that may have signaling functions involved in regulating the degree of paracellular permeability.¹⁵³

Structural analysis of the zonula occludens by electron microscopic observation of freeze-fracture

replicas of the plasma membrane has shown that tight junctional proteins (occludin) assemble in linear strands or fibrils within the plasma membrane (Figs 3-15a and 3-15b). The plasma membranes of the adjacent cells are fused along the linear strands of occludin proteins. The zonula occludens contains multiple anastomosing tight junctional strands.

Physiologic studies of epithelial permeability have shown that there is no clear correlation between the number and arrangement of tight junctional strands and the degree of intercellular occlusion. Some zonula occludens act as total barriers, while others (leaky tight junctions) permit the flow of ions and solutes through the paracellular space. Modulation of the contraction of the actomyosin ring (terminal web) associated with the zonula occludens and zonula adherens has been proposed as an explanation for differences in tight junctional permeability. Contraction of the actomyosin ring mediated by myosin light-chain kinase exerts tension on components of the zonula occludens, thereby altering the permeability of the paracellular space.¹⁵⁴

The presence of a zonula occludens at the distal end of the secretory ameloblast, just proximal to Tomes process, suggests that the space into which the enamel matrix is deposited is isolated from the intercellular spaces of the enamel organ. The enamel mineralization compartment is bounded below by

Fig 3-16 Microtubule assembly by parallel association of tubulin protofilaments. Each protofilament forms by binding heterodimers of α and β tubulin at the positive end of the microtubule. Heterodimers are added when they are in the guanosine triphosphate (GTP)-bound state.

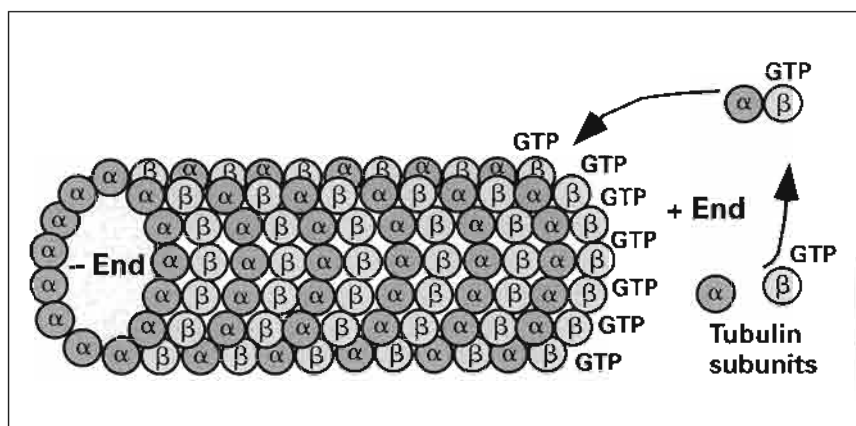
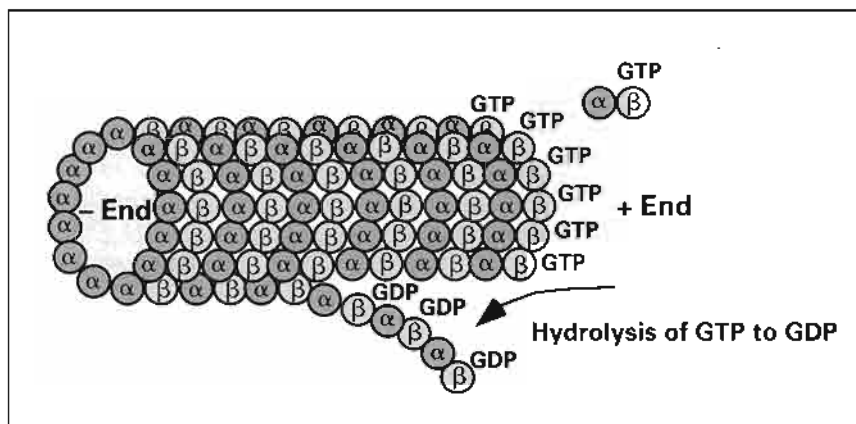


Fig 3-17 Hydrolysis of guanosine triphosphate (GTP) to guanosine diphosphate (GDP). Hydrolysis of GTP on the β tubulin subunit destabilizes the protofilaments, leading to rapid depolymerization of microtubules.



mineralized dentin and above by the ameloblasts joined together by zonula occludens junctions. Analysis of the fluid contained in this compartment indicates that it has a different composition than the general extracellular fluid and serum.

In addition to creating an intercellular barrier, the zonula occludens of the secretory ameloblast may stabilize the secretory domain of Tomes process (analogous to the development of a luminal membrane compartment in other polarized secretory cells). The zonula occludens of the ruffle-ended ameloblast may have a similar role in maintaining the ruffled border and sealing the intercellular space of the enamel organ from the enamel compartment.

Microtubules and motor proteins in secretion

Microtubules (MTs) form a key component of the cytoskeleton in all cells. They provide a scaffold on which organelles, vesicles, and secretory granules are translocated by the action of motor proteins. In addition,

MTs act as rigid struts involved in maintaining cell shape. During mitosis, MTs assemble to form the spindle apparatus required for chromosomal segregation.

Each MT is a hollow cylinder constructed of 13 protofilaments of tubulin. Tubulin protofilaments are assembled from heterodimers of α and β tubulin molecules (Fig 3-16).¹⁵⁵ The addition and removal of tubulin heterodimers takes place at opposite ends of an MT. The positive (+) end of an MT is the growing end, while the negative (-) end is the point of removal of tubulin. The removal of subunits at the negative end of an MT is slower than the rate of addition of new subunits at the positive end.

Both α and β tubulins are guanosine triphosphate (GTP)-binding proteins. In the GTP-bound state, the β tubulin subunit has a high binding affinity, thereby favoring rapid addition of subunits at the growing end of the elongating protofilament.¹⁵⁶ Hydrolysis of GTP on the β tubulin subunits destabilizes the protofilament structure, causing rapid depolymerization of the MT (Fig 3-17). Microtubules continue to grow as long as the rate of addition of GTP tubulin is faster than the rate of GTP hydrolysis.

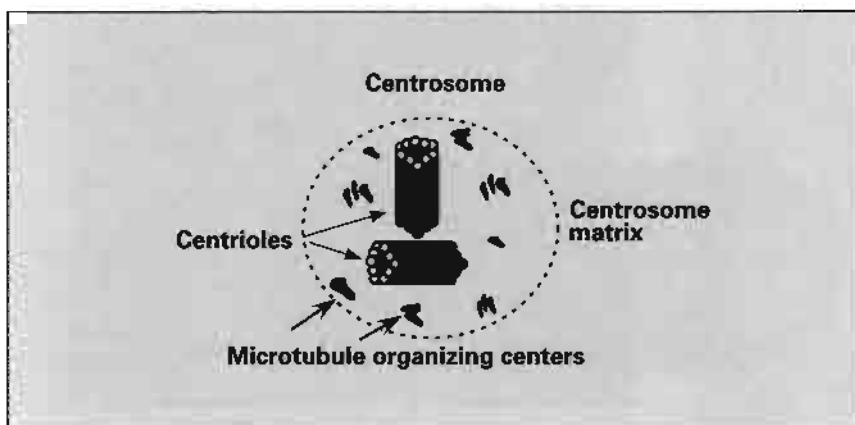


Fig 3-18 Microtubule organizing centers. Numerous microtubule organizing centers are located in the centrosomal matrix associated with the centrioles. Each microtubule organizing center nucleates the development of a microtubule and stabilizes the microtubule by capping the negative end.

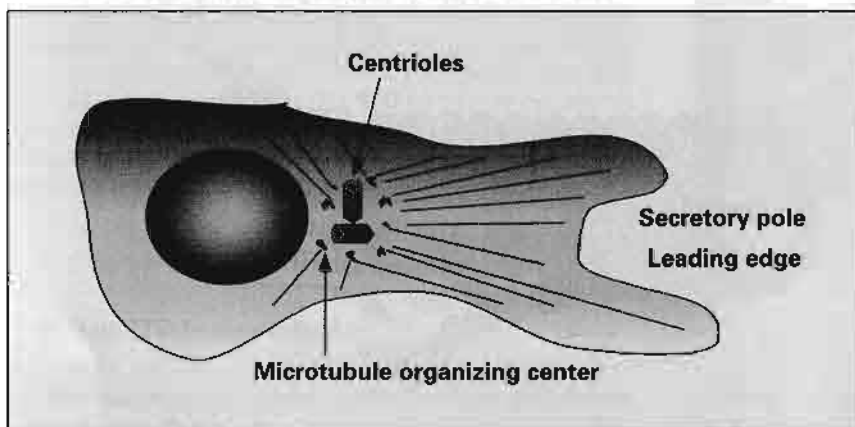


Fig 3-19 Centrosome, consisting of a pair of centrioles and associated microtubule organizing centers. The centrosome regulates the polarization of the cellular microtubule network. The positive ends of the microtubules are located in the peripheral cytoplasm.

Initiation of the polymerization of an MT requires the action of the microtubular organizing center (MTOC). The composition and mechanism of action of the MTOC is poorly understood. A third form of tubulin, γ tubulin, is found in MTOCs, where it performs a nucleating function. The most prominent MTOC is associated with the centrioles. Numerous MTOCs are located in the cytoplasm (the pericentriolar matrix) surrounding each pair of centrioles (Fig 3-18).

The negative end of the growing MT is stabilized by components of the MTOC. This arrangement permits the polarized growth of MTs away from the MTOC and toward the peripheral cytoplasm (Fig 3-19). Microtubules radiate from the centrosome outward toward the plasma membrane, where the positive end of each MT is capped by special proteins.

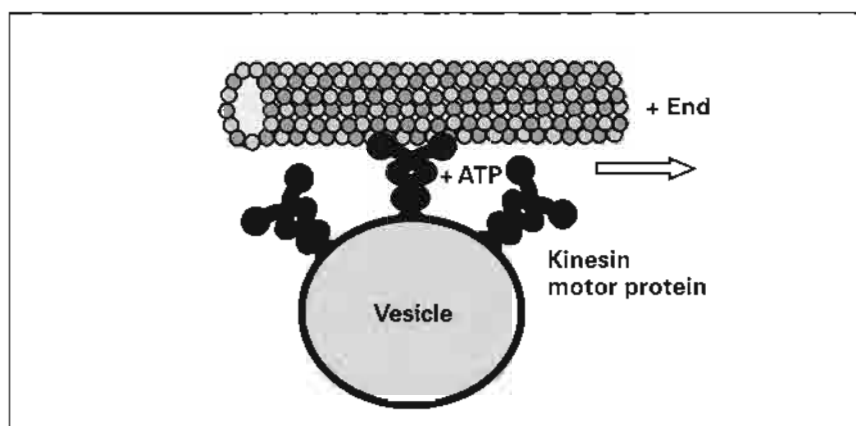
Microtubules are stabilized by interaction with capping proteins, microtubule-associated proteins, and by detyrosination (removal of tyrosine from the carboxy terminal of tubulin). Detyrosinated MTs constitute a small percentage of the total microtubular complement of the cell. They have a life span of

about 2 hours. Most MTs are unstable. Unstable MTs are dynamic structures whose average life span is about 10 minutes.

Microtubules serve as conduits for the transport of organelles and vesicles.¹⁵⁷⁻¹⁵⁹ Transport requires the action of microtubule-associated motor proteins (motor MAPs) and ATP. The most widely studied motor MAPs are the kinesin and dynein families of motor proteins. Classic kinesin is composed of two heavy chains and two light chains.¹⁶⁰ The heavy chain contains a large N-terminal globular head group with binding sites for ATP and tubulin. The tail portions, stabilized in a helical conformation, contain binding sites for various integral membrane proteins that are contained in the limiting membranes of organelles, vesicles, and granules (Fig 3-20). Dynein is a multimeric complex of heavy, intermediate, and light chains.

Motor MAPs transform the chemical energy released by the hydrolysis of ATP to adenosine diphosphate into mechanical displacement of the motor protein and its cargo along the surface of the MT (see Fig 3-20). It is unclear whether the movement of the motor protein and its cargo is caused by a con-

Fig 3-20 Association of kinesin motor protein to a microtubule and the limiting membrane of a cytoplasmic vesicle. Adenosine triphosphatase (ATP) activity in the globular head group of the motor microtubule-associate protein causes displacement of the vesicle toward the positive end of the microtubule.



formational change (ratchet power stroke) in the motor protein or by some form of biased diffusion along the MT surface. In general, kinesin transports cargo from the centrosome toward the peripheral cytoplasm, while dynein transports cargo in the opposite direction. For example, dynein has been shown to be needed for early endosome to late endosome and lysosomal transport, while kinesin is necessary for the transport of secretory granules from the trans-Golgi network to the plasma membrane.

Individual members of these two families of motor MAPs appear to be relegated to the movement of specific cytoplasmic organelles and inclusions, for example, secretory granules, mitochondria, and transport vesicles. Specificity is believed to be a function of the binding affinity of carboxy tail domains of the motor MAP to target (receptor) proteins in the membrane of the transported entity.

Microtubule-associated structural proteins (structural MAPs) help to stabilize MTs by forming bridges to other cytoplasmic proteins.^{156,161} They are present in high numbers in axons and dendrites of nerve cells. Approximately 15% to 20% of the total proteins of the brain are made of tubulin and MAPs.

Because of the abundance of structural MAPs in the brain, the neuronal MAPs have been most highly studied. Neuronal MAPs stabilize and promote the alignment of MTs in parallel arrays in axons and dendrites. In nonneuronal cells of the body, MTs are stabilized and bundled by MAP4. Phosphorylation of serine and threonine residues on MAPs by various kinases leads to MAP inactivation and decreased ability to interact with tubulin. On the other hand, the action of various phosphatases can activate MAPs and increase the organization of MT systems.

Clinical Correlations

Enamel dysplasia

Alteration of the ionic and metabolic milieu of secretory and maturation ameloblasts leads to defective enamel (*enamel dysplasia*). Because enamel does not remodel, its defects are retained in the fully formed tooth. Interference with the secretion stage leads to a reduction in the amount and/or composition of the enamel matrix, a condition known as *enamel hypoplasia*. The resulting enamel is thinner than normal, but usually fully mineralized. If the interference affects maturation ameloblasts, the result will be hypomaturation and hypomineralization. The resulting enamel contains more protein than normal, and the hydroxyapatite crystallites fail to reach their normal size.

Early maturation appears to be a critical stage in the formation of sound enamel, because disturbances occurring during the period when transitional ameloblasts differentiate into maturation ameloblasts lead to prolonged periods of suboptimal mineralization.¹⁶² Differentiating and secretory ameloblasts appear to have a greater potential for recovering normal function following metabolic insult.

Enamel affected by hypomaturation is usually of full thickness but more porous and less mineralized. Because it is less translucent, hypomaturation enamel appears clinically as white spots, or it may appear stained because of the subsequent absorption of extraneous molecules derived from food and serum. Dysplastic enamel usually contains physical evidence that both matrix deposition and maturation have been altered. Such teeth may have horizontal rows of pits and grooves of discolored and white opaque enamel.

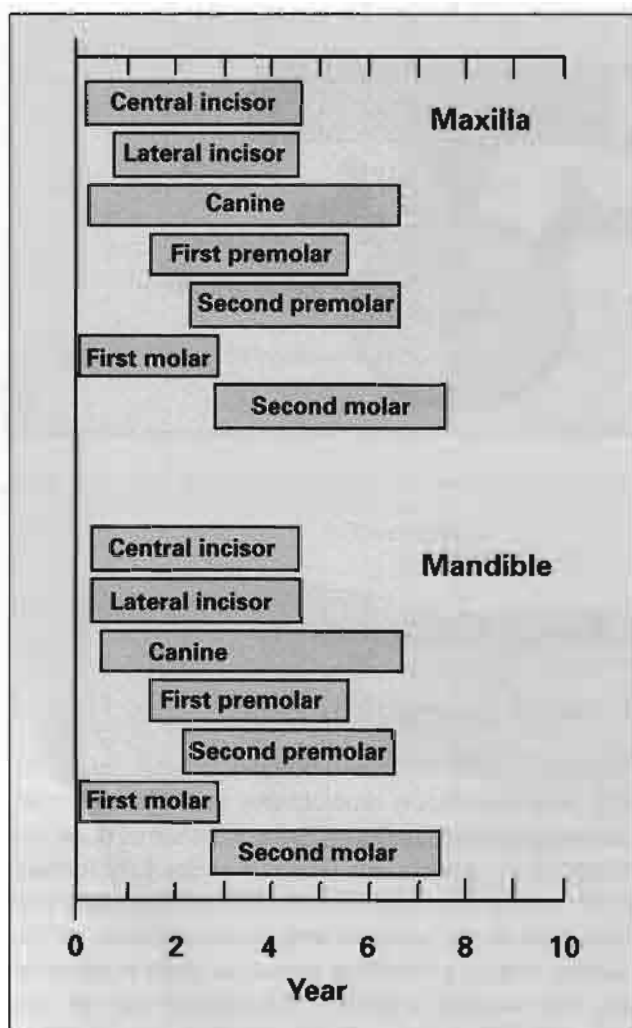


Fig 3-21 Period of amelogenesis in the permanent teeth of the human dentition. Each bar represents the duration of enamel formation from beginning to completion of maturation. (Adapted from Seltzer and Bender.¹⁶³)

Enamel dysplasia can be caused by local, systemic, and hereditary factors:

1. Anoxia in premature birth
2. Congenital syphilis
3. Erythroblastosis fetalis
4. Exantematous infections
5. Fluorosis
6. Hypoparathyroidism
7. Hypothyroidism
8. Renal hypophosphatasia
9. Vitamin A deficiency
10. Vitamin D-resistant rickets

In locally acting etiologies, there is no regular pattern involving contralateral teeth and no consistent

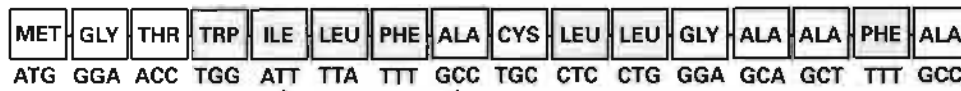
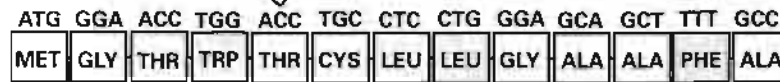
pattern with relation to timing. An example of a local factor is an inflammatory process in a carious primary tooth that affects the nearby dental germ of its permanent replacement.

In a patient with enamel defects, symmetry of the lesions usually indicates a systemic cause. Systemic agents act in a symmetric and contemporaneous manner to involve all teeth under development at the time of the insult. Based on the position, distribution, and nature of the lesions, the approximate time period over which a disease occurred can be determined. The chronology of enamel formation (Fig 3-21) reveals how a serious systemic disease, such as pneumonia or measles, affecting a 1-year-old child will cause enamel hypoplasia of the permanent incisors, canines, and first molars.¹⁶³ A similar disease occurring in a 3-year-old child will affect the maturation phase in the incisors and canines and the secretory phase of the premolars. Recurrent systemic diseases, or the medications used in their treatment, frequently produce a series of symmetric horizontal ridges and grooves across the enamel surface.

Enamel defects caused by environmental factors are not uncommon. In a study of more than 1,500 schoolchildren in London, it was reported that 68% had enamel defects in the permanent dentition.¹⁶⁴ More than 10% had defects on 10 teeth or more.

Genetically acquired enamel defects are much rarer than the environmentally produced varieties. Hereditary enamel dysplasia, also known as *amelogenesis imperfecta*, occurs in several forms. The hypoplastic form, involving the secretion stage, leads to thin enamel. The teeth are smaller and lack contact points. Exposure of dentin and hypersensitivity are common sequelae. In the hypocalcified or hypomatured type, the enamel is soft, deeply stained, and easily chipped away from the dentin. In general, affected enamel shows an inverse relationship between mineral and protein contents.¹⁶⁵

Amelogenesis imperfecta may be inherited as an autosomal-dominant defect with variable penetrance or as a sex-linked dominant trait. It was recently shown that a mutation in the *AMEX* gene, deleting nine base pairs in exon 2, resulting in the loss of three amino acids and the exchange of one amino acid in the signal peptide of amelogenin, was sufficient to cause severe enamel hypoplasia (Fig 3-22).¹⁶⁶ In yet another family, a mutation on the *AMEX* gene, leading to the deletion of a much larger segment (5 kilobases) and the loss of entire exons, caused hypomineralization of enamel (Figs 3-22 and 3-23).¹⁶⁷

Normal**Mutant**

☐ Hydrophobic amino acid
 ☐ Neutral amino acid
 ☐ Polar amino acid

Fig 3-22 Base pair and amino acid sequences of normal and mutant signal peptide portions of the human *AMEX* gene and amelogenin protein. Mutation leading to the loss of a tripeptide (isoleucine, leucine, and phenylalanine) and the substitution of threonine for alanine cause severe hypoplasia. (Adapted from Lagerström-Fermér et al¹⁶⁷ with permission.)

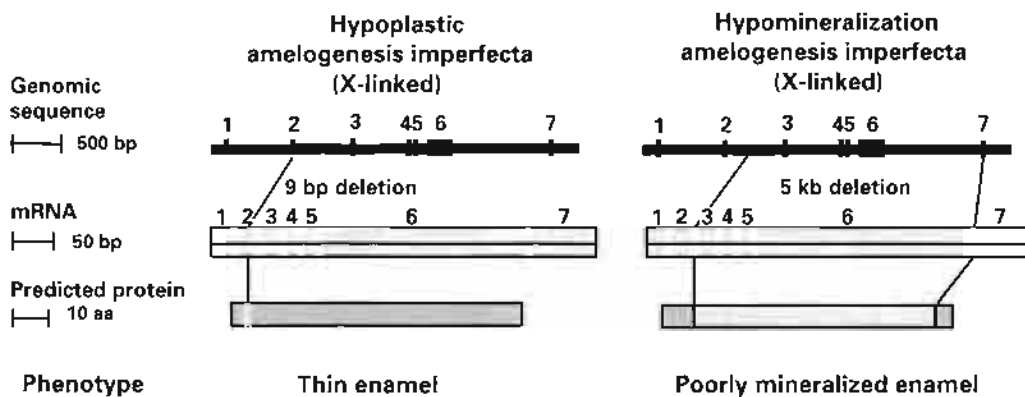


Fig 3-23 Two mutations on the *AMEX* gene that cause amelogenesis imperfecta. (Adapted from Lagerström-Fermér et al¹⁶⁷ with permission.)

Enamel pits and fissures

During the development of multicusped teeth, pit and fissure defects are formed in the steep depressions separating adjacent cusps. These defects form when the enamel organ is compressed by the growth of enamel along the slope of the cusps, constricting the ameloblasts that are located in the deepest and most narrow regions of the depression (Fig 3-24). Degeneration of the ameloblasts results in the formation of a pit and/or fissure running from the surface of the crown to a level just above the dentin. A thin layer of enamel at the base of the defect is usually formed by the ameloblasts prior to their death. The space created by the degeneration of the cells of the enamel organ provides a niche that becomes colonized by bacteria as soon as the tooth erupts into the mouth.

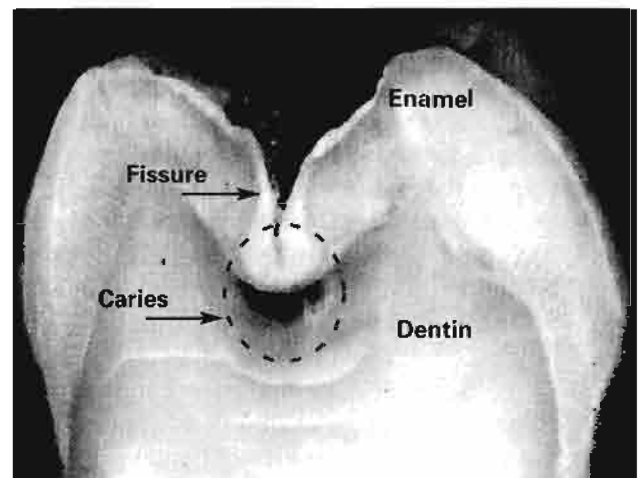


Fig 3-24 Mineralized tooth sectioned in half to reveal caries in the enamel (chalky white) along the sides of a fissure and below it in the dentin (brownish red). (From Paterson et al.¹⁷² Reprinted with permission.)

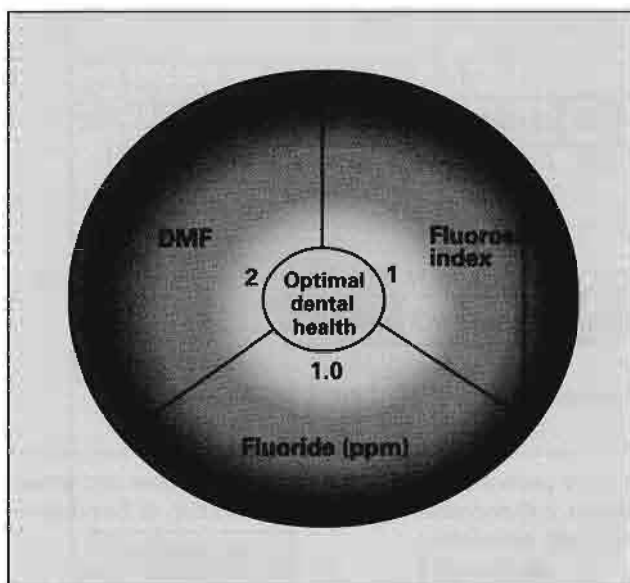


Fig 3-25 Relationship between fluoride levels in drinking water (in parts-per-million [ppm]), the detrimental effects of high fluoride intake (fluorosis index), and dental health as measured by the number of diseased, missing, and filled teeth (DMF). (Adapted from Shaw et al.¹⁷⁰)

Pits and fissures are the parts of the tooth most susceptible to caries attack. Acid production by bacteria demineralizes adjacent enamel and dentin, leading to the formation of an incipient carious lesion (see Fig 3-24). Because the amount of enamel at the floor of the defect is minimal, the caries process can invade the dentin within a short time after its initiation. Unless teeth are protected with fluoride or an occlusal sealant, there is a very high probability that they will develop clinically detectable pit and fissure caries within 2 years after eruption.

Infant malnutrition and dental disease

A growing body of evidence accumulated during the past two decades has established the importance of an adequate intake of protein during early childhood for optimal dental health. Protein malnutrition during the formative years leads to delay of tooth eruption and increased susceptibility to dental caries later in life.^{168,169} The combination, frequently found in underdeveloped countries, of protein malnutrition in infants and subsequent increased consumption of sucrose-containing foods by children and adolescents, leads to a widespread incidence of dental caries.

Although the specific underlying biochemical deficiencies in enamel and dentin matrix that result from insufficient consumption of dietary protein remain to be established, it is clear that ameloblasts

and odontoblasts require high levels of energy as well as a wide variety of amino acids for protein synthesis. Thus they are detrimentally affected in periods of protein starvation. Furthermore, enamel matrix produced during embryonic development and early childhood cannot undergo subsequent remodeling. Therefore, any deficiency occurring during its development will lead to increased susceptibility to dental disease in later life.

Fluoride, dental caries, and fluorosis

Epidemiologic studies conducted nearly a half-century ago demonstrated an inverse relationship between the level of fluoride in local water supplies and caries experience. Children who grew up in communities with fluoride levels greater than 1.0 ppm in drinking water experienced significantly fewer dental caries than did children in neighboring towns with low fluoride concentrations (less than 0.5 ppm) (Fig 3-25).¹⁷⁰

Fluoride enters hydroxyapatite mineral, where it substitutes for hydroxyl ions. Incorporation of fluoride into enamel and dentin occurs during tooth development. Additional fluoride is added even after enamel maturation, as fluoride is absorbed in surface enamel from tissue fluids prior to eruption and from saliva once the teeth have erupted into the oral cavity. Fluoridated hydroxyapatite is more resistant to acid demineralization than is nonsubstituted mineral. The addition of only small amounts of fluoride to the hydroxyapatite crystal greatly improves its stability by decreasing the mobility of hydroxyl ions within the crystal lattice.¹⁷¹

The addition of fluoride in drinking water supplies at the optimal level of 1.0 ppm has a great dental health benefit, reducing the incidence of dental caries by more than 50%. Furthermore, the reduction in dental caries minimizes other pathologic sequelae that result from early tooth loss.

High levels of fluoride consumed during the period of tooth development have detrimental effects on enamel formation (see Fig 3-25). Sustained consumption of fluoride at levels greater than 4.0 ppm causes dental fluorosis, a condition characterized by chalky white defects and areas of yellow-brown discoloration in the enamel. Chronic ingestion of fluoridated toothpaste or mouthrinse in areas with optimally fluoridated water can raise systemic fluoride concentrations to a level at which fluorosis may develop. Despite the enamel defects, the involved teeth are more resistant to caries. For the patient, enamel fluorosis is mainly an esthetic problem.

Analysis of fluorotic enamel indicates that it is more porous and less mineralized. There is also evidence of retention of some amelogenin protein, indicating a defect in maturation, possibly related to the inhibition of the proteolytic breakdown of enamel proteins. In addition, ultrastructural studies of ameloblasts indicate that fluoride can disrupt the secretory phase by obstructing the normal vesicular transport of secretory proteins.

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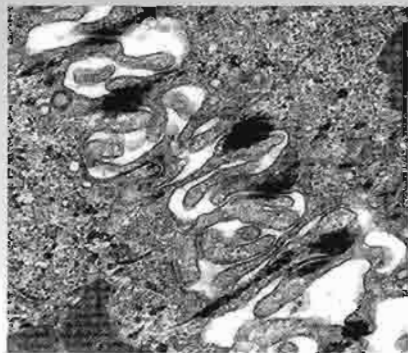
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Chapter 4

Oral Mucosa



Traditionally, oral mucosa is divided into three categories: lining mucosa, masticatory mucosa, and specialized mucosa (Fig 4-1 and Table 4-1).^{1,2} Lining mucosa is distensible and relatively loosely bound to its adjacent structures by connective tissue that is rich in elastin. It is found over mobile structures such as the lips, cheeks, soft palate, alveolar mucosa, vestibular fornix, and the floor of the mouth. Masticatory mucosa is the protective-covering component of the gingiva and hard palate. It is rigid, tough, and tightly bound by dense connective tissue to underlying bone. Specialized mucosa is located on the dorsum of the tongue. It contains specialized mucosal structures: the lingual papillae and taste receptors. The heterogenous pattern of keratin expression in the tongue is complex and in part is responsible for generating the papillary architecture of the lingual epithelium.

Oral mucosa is made up of stratified squamous epithelium (SSE) and underlying connective tissue consisting of a lamina propria and submucosa. Papillary projections of lamina propria connective tissue indent the epithelium and support fine nerve fibers and terminal vascular loops. The size and number of connective tissue papillae vary with each region of the oral mucosa. Increased convolution of the junction between epithelium and connective tissue appears to be directly related to the degree of mechanical stress exerted at a particular mucosal site.³ The submucosa is located between the lamina propria

and underlying bone and muscular tissue (Fig 4-2). It contains glands, adipose tissue, neurovascular bundles, specialized nerve endings, and lymphatic tissues.

Cell Proliferation and Differentiation in Stratified Squamous Epithelia

Stratification of the SSE is the result of cell proliferation and sequential differentiation. Proliferation is a property of stem cells of the basal cell layer and their immediate progeny, the transit-amplifying cells. Differentiation starts when recently divided cells detach from the underlying extracellular matrix. The regulatory signals that must be activated to initiate keratinocyte differentiation are clearly complex. Detachment of the $\alpha 6 \beta 4$ integrin to its extracellular ligand (laminin 5) is one of the switches that must be triggered for keratinocyte differentiation and cell stratification.⁴ As differentiating cells mature, they are pushed toward the epithelial surface by pressure generated in the underlying proliferation compartment.

Terminal differentiation of SSE follows either one of two major pathways. Fully cornified dead cells (squames) are formed in the epidermis, hard palate, and oral gingival epithelium (OGE). In contrast, in lin-

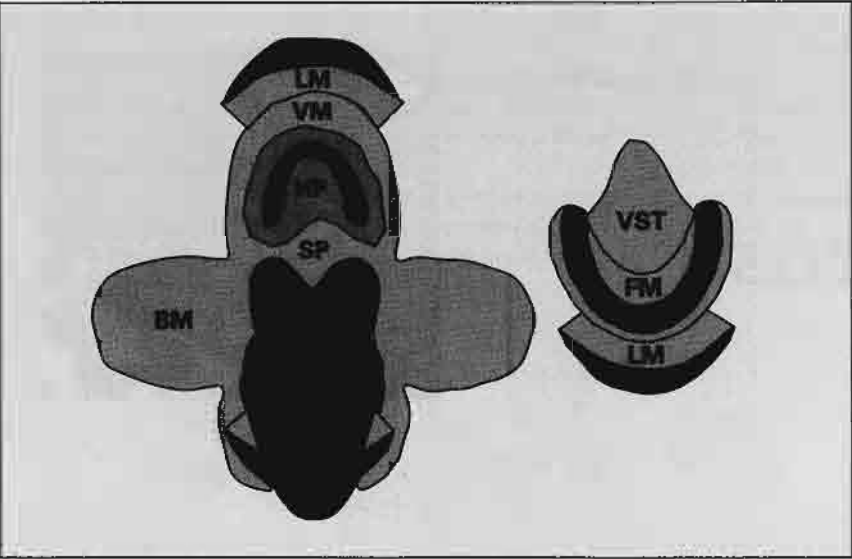


Fig 4-1 Location of various differentiation patterns in oral mucosa. Masticatory mucosa (cornified) is located over the hard palate (HP) and the gingiva (GM). Non-cornified lining mucosa lines the buccal surfaces (BM), the soft palate (SP), the vestibular surfaces (VM), the ventral surface of the tongue (VST), and the floor of the mouth (FM). Labial mucosa (LM) and the vermillion border (VB) are covered by a variation of lining mucosa. The dorsum of the tongue (DT) is covered by specialized mucosa containing filiform, fungiform, and circumvallate papillae and numerous taste buds. Alveolar mucosa, a form of lining mucosa extending from the base of the gingival mucosa and merging with the vestibular mucosa, is not shown. (Adapted from Roed-Petersen and Renstrup,² with permission from Taylor and Francis.)

Table 4-1 Types of oral mucosa			
Characteristic	Lining mucosa	Masticatory mucosa	Specialized mucosa
Surface	Noncornified	Cornified/parakeratinized	Cornified and noncornified
Submucosa	Loose connective tissue	Dense connective tissue	Loose connective tissue
Fibers*	Collagen fibers + Elastic fiber +++	Collagen fibers +++ Elastic fibers +	Collagen fibers ++ Elastic fibers ++
Sensory receptors	Merkel cells Nociceptors Encapsulated mechanoreceptors	Merkel cells Nociceptors Encapsulated mechanoreceptors	Taste buds Nociceptors Encapsulated mechanoreceptors

* Concentration of fibers is indicated as low (+), medium (++), or high (+++).

ing mucosa, the outer-level cells are noncornified. Site-specific differentiation also gives rise to epithelial appendages, such as the filiform papillae of the tongue.

Proliferation and differentiation are controlled by autocrine and paracrine factors generated by the keratinocytes, cytokines and growth factors originating in the underlying connective tissue, and circulating systemic factors (see “Control of keratinocyte differentiation,” later in this chapter).⁵⁻⁸

Numerous studies have documented the importance of gap junctional communication between epithelial cells in regulating differentiation.^{9,10} Cell-to-cell transfer of nutrients and small regulatory substances across gap junctions may play an especially important role in maintaining the orderly devel-

opment in this avascular tissue. Physiologic hyperplasia, as induced by the application of retinoic acid, increases the expression of gap junction protein and cell-to-cell communication.¹¹ In contrast, pathologic hyperplasia and metaplasia are usually accompanied by a reduction in gap junctional communication (see “Squamous cell malignant transformation,” later in this chapter).

The proliferation compartment contains two pools of dividing cells: stem cells and transit-amplifying cells (Fig 4-3). Stem cells located in the basal cell layer are slow-cycling cells. They are not distributed homogenously in the basal layer but are grouped in a pattern set by cell-to-cell communication among keratinocytes.^{12,13} It has been suggested that the presence of keratin 19 (K19) and the expression of

Fig 4-2 Components of the oral mucosa. Stratified squamous epithelium (E) rests on the lamina propria (LP), a zone of well-vascularized, relatively loose connective tissue. Depending on the site, the submucosa (SM) may contain fat deposits (F) and minor salivary glands (SG). The submucosa covers deeper muscular tissue (M) or, in the gingiva and hard palate, rests directly on bone.

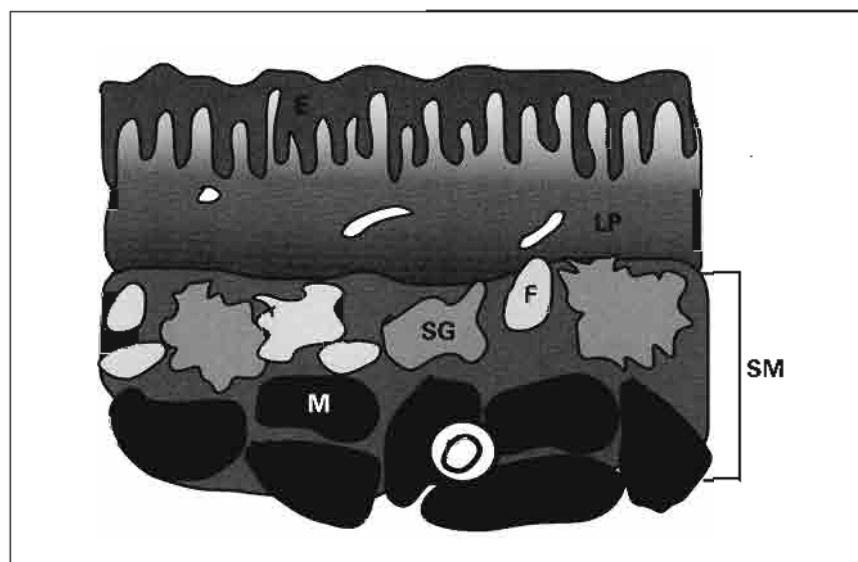
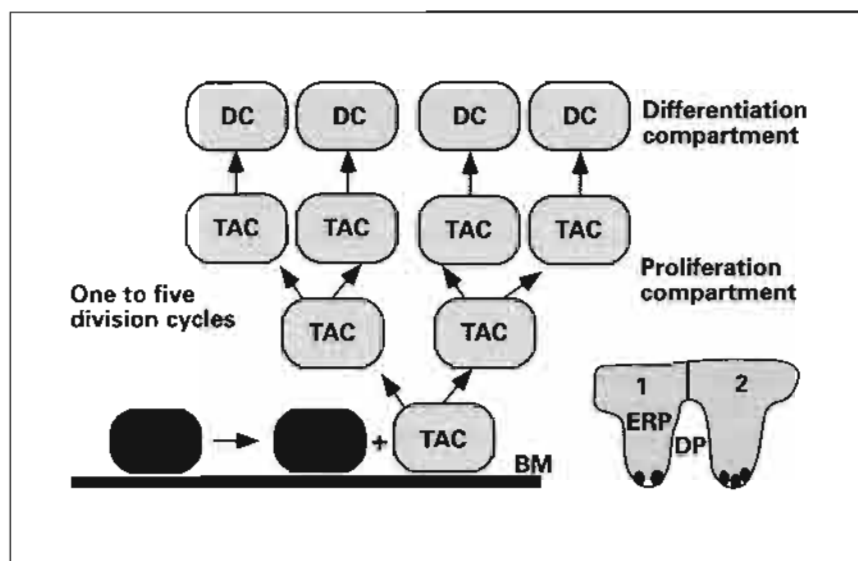


Fig 4-3 Components of an epidermal proliferation unit (EPU). In the proliferation compartment, stem cells (SC) located adjacent to the basal lamina divide to give rise to transit-amplifying cells (TAC). They subsequently divide to produce a clone of differentiating cells. In the differentiation compartment (DC), keratinocytes mature, acquiring the characteristics of the various strata as they move to the surface. The inset shows the location of two EPU's, demarcated by a vertical line bisecting the epithelium overlying the crest of the dermal papilla (DP). (BM) Basement membrane; (ERP) epithelial rete peg.



$\beta 1$ integrins may serve as biochemical markers of stem cells.¹³ Transit-amplifying cells, derived from the division of stem cells, divide one to five times while migrating laterally and upward toward the epithelial surface, producing a clone of differentiating cells.¹⁴

Dividing cells and their respective clones of differentiating cells form epidermal proliferation units (see Fig 4-3).^{14,15} Each epidermal proliferation unit consists of a cohort of differentiating keratinocytes originating from daughter (transit-amplifying) cells. Cells with the highest potential for cell division reside in the deepest portions of the epithelium, ie, at the

base of the epithelial ridge (Figs 4-3 and 4-4).¹³ This pattern of stem cell distribution is found in thick skin and oral mucosa (eg, the dorsal surface of the tongue). The concept of the epidermal proliferation unit is clearly demonstrated in the organization of keratinocytes of filiform papillae in mice, where they occupy an area about 40 μm in diameter along the basement membrane of the dorsal mucosa of the tongue. In the larger filiform papillae of humans, there are many more epidermal proliferation units, with a more complex organization, yet the same principle of basal proliferating cells giving rise to cohorts of differentiating cells is applicable.

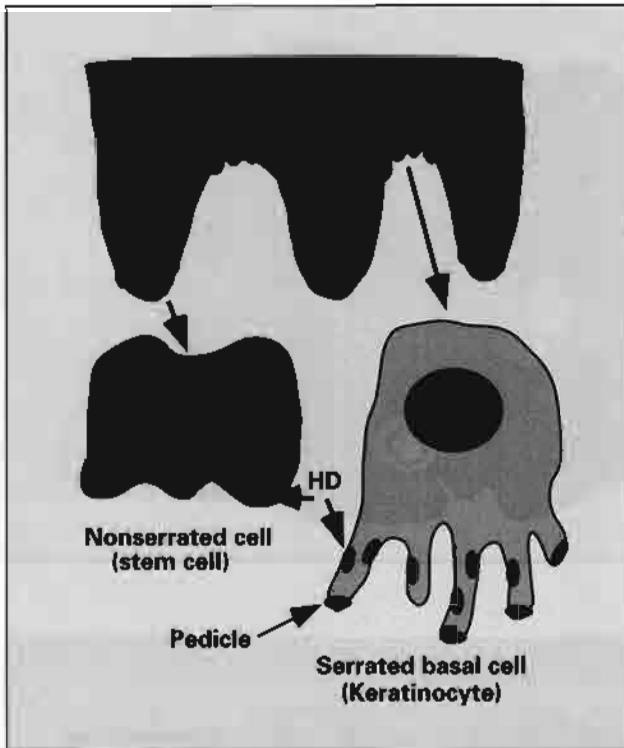


Fig 4-4 Differentiation of basal cells. Basal cells located at the deepest point of the epithelial pegs (EP) or ridges are small and have a less convoluted shape. These nonserrated cells are less specialized for attachment and have the highest potential for cell division. Basal cells located over the apex of the connective tissue papillae have a serrated surface because of the numerous cytoplasmic processes (pedicles), rich in hemidesmosomes (HD), specialized for anchorage to the extracellular matrix. In the oral cavity, there is considerable variation from site to site in the degree of basal cell–connective tissue interdigitation; thus, the development of the serrated phenotype is not uniformly present.

Epithelial turnover increases during the healing of wounds. Proliferation can be increased by an increase in the number of stem cells that are dividing, by a shortening of the cycle time of stem cells and transit-amplifying cells, or by an increase in the number of times the transit-amplifying cells divide.¹⁶ Safety mechanisms, basic to all cell types, monitor the genetic material of epithelial stem cells to minimize the survival and proliferation of damaged cells.

In the oral cavity, SSE follows one of two major differentiation pathways: full cornification, as found on the hard palate and gingiva, or noncornification as on the buccal mucosa. Before the specific aspects of oral mucosal surfaces are considered, it is necessary to review epidermal differentiation.

Structure and Function of the Cornified (Orthokeratinizing) Epithelium: The Epidermal Model

Epidermal biology is a rapidly growing branch of biomedical science of special significance to oral biology. Much of the molecular biology of epidermal differentiation is highly relevant to understanding cellular function in oral stratified squamous epithelium. The following is a brief review of epidermal cellular structure and function.

Basal cells

In skin and other cornified SSE, differentiating cells form morphologically distinct strata: the basal cell layer, the stratum spinosum, the stratum granulosum, and the stratum corneum.

In all SSE, basal cells are supported by a basement membrane, a specialized extracellular molecular network, constructed jointly by epithelial and connective tissue cells. This narrow strip of specialized extracellular matrix can be observed in the light microscope when stained by dyes that bind to negatively charged residues of its glycosaminoglycans and acidic glycoproteins. In routine sections stained with hematoxylin and eosin, the basement membrane appears as a pink-to-purple band approximately 0.5 μm thick. With the introduction of electron microscopy, the basement membrane was observed to consist of special collagen fibrils and a 50- to 60-nm-thick matlike structure, the basal lamina, located just beneath all basal cells of healthy epithelial tissues.

In tissues prepared for routine electron microscopy, a dense matlike component, the lamina densa, appears to be separated from the epithelial cell membrane by a clear zone, the lamina lucidum. Recent studies have shown that the lamina lucidum is a preparation artifact produced during tissue dehydration. In reality, the basal lamina consists solely of a lamina densa in direct juxtaposition to the cell membrane. The composition of the basal lamina densa and its role in anchoring epithelium to connective tissue is discussed in the section, "Basal attachment apparatus," later in this chapter.

The basal cell layer contains a small pool of stem cells and a larger pool of basal cells whose function is to anchor the epithelium to the connective tissue. Basal cells have a cuboidal to high-cuboidal shape. Their nuclei are round to ovoid and frequently located furthest from the basal lamina, giving the cell a slightly polarized appearance. Cisternae of

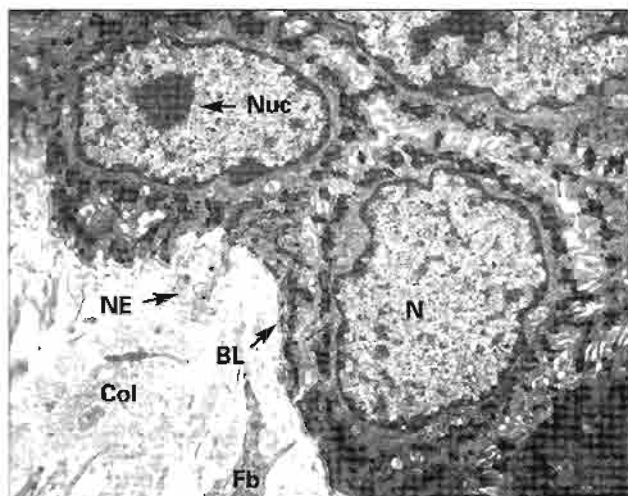


Fig 4-5 Transmission electron micrograph of nonserrated basal cells. (BL) Basal lamina; (N) nucleus; (NE) nerve ending; (Nuc) nucleolus; (Col) collagen; (Fb) fibroblast. (Original magnification $\times 4,500$.)

rough endoplasmic reticulum (RER), Golgi saccules, mitochondria, and polyribosomes constitute a relatively small component of the cytoplasm of the basal cell. Filaments comprising K5 and K14 keratin chains occupy roughly 25% of the cytoplasmic volume.

Basal cells synthesize and secrete type IV and type VII collagens, laminin, perlecan, parathyroid hormone-related peptide, and cytokines. The cytoplasmic volume of keratinocytes doubles during differentiation to form the upper layers of the stratum granulosum. Although the rate of protein synthesis increases and the total protein more than doubles during keratinocyte differentiation, the ratio of keratin to nonkeratin protein remains constant.¹⁷ As the cells differentiate, the nucleus-to-cytoplasmic ratio decreases.

Basal cells situated along the base of the epithelial ridge (rete peg) have a relatively flattened cell surface in contact with the connective tissue (Fig 4-5). These nonserrated basal cells contain only a few cytoplasmic organelles and appear to be the least differentiated cells in the epidermis. The nonserrated cells represent the stem cells and transit-amplifying cells.^{12,13} They are characterized by a high nucleus-to-cytoplasmic ratio, expression of K19, a relative lack of keratin filament bundles, and high levels of $\beta 1$ integrins.^{12,13}

Stem cells of the epidermis contain melanin pigment as a result of their close association with melanocytes. Another characteristic of stem cells is the expression of Bcl-2 protein, an inhibitor of apoptosis (see chapter 13).¹⁸

When a stem cell divides, one daughter cell retains the properties of the stem cell population, while the

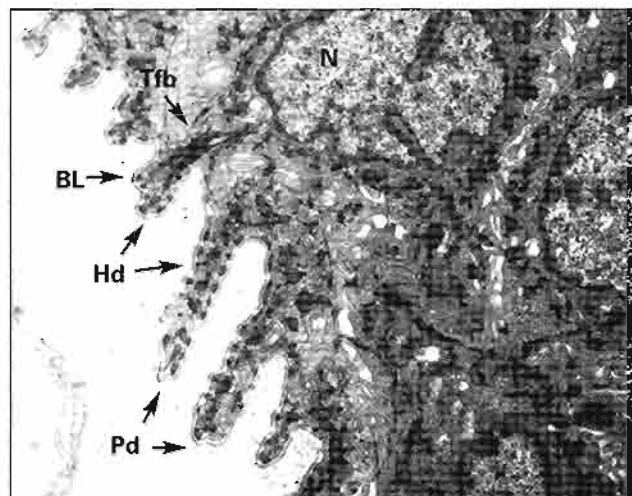
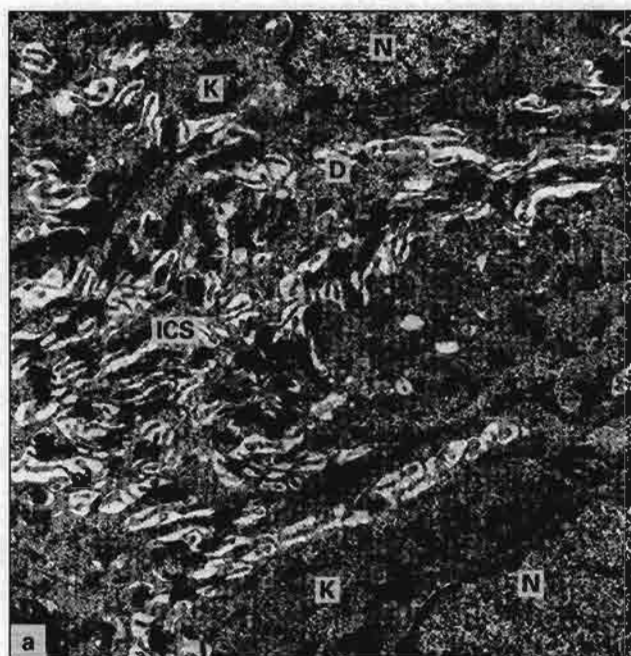


Fig 4-6 Transmission electron micrograph of serrated basal cells. (BL) Basal lamina; (N) nucleus; (Pd) pedosomal processes; (Tfb) tonofilament bundle; (Hd) hemidesmosomes. (Original magnification $\times 4,500$.)

other daughter cell enters the transit-amplifying cell pool. The conversion from stem cell to transit-amplifying cell is driven by the expression of the transcription factor c-Myc.¹³ Some proliferative transit-amplifying cells may be found in the cell layers immediately adjacent to the basal cells. Transit-amplifying cells have lower levels of $\beta 1$ integrin expression.¹³

At the apex of the dermal papillae, along the thinnest portion of the epidermis, many basal cells have a serrated basal surface in contact with the basement membrane (Fig 4-6). Numerous cytoplasmic processes (pedicles) that project into the underlying connective tissue create the serrated appearance. These basal cells appear specialized for anchoring the epidermis to the connective tissue. The pedicles are rich in hemidesmosomes and have well-developed filament bundles terminating at the attachment plaques. In thymidine labeling studies, nonserrated basal cells at the base of epithelial ridges were heavily labeled, while serrated basal cells were weakly labeled, confirming the notion that the stem cell pool correlates with the nonserrated cell type.

The position of the stem cell pool at the base of the rete peg does not hold true for all parts of the epidermis. In scalp epithelium, the stem cells are located over the crest of the connective tissue papillae, just the opposite of the condition found in thick planar epithelium.¹³ In vitro studies show that pattern generation, ie, the regular spacing of epidermal proliferation units, is an internal function of keratinocytes, independent of connective tissue influence.¹³



Figs 4-7a and 4-7b Electron micrographs of the stratum spinosum layer. (a) Intercellular spaces (ICS) between several keratinocytes (K) demonstrating the abundance of desmosomes (D). (N) Nucleus. (Original magnification $\times 5,100$.) (b) High magnification of intercellular spaces with desmosomes (D), adherens junction (Aj), and gap junction (Gj). (Kf) Keratin filaments. (Original magnification $\times 38,000$.)

Stratum spinosum

The stratum spinosum forms the first layer of the differentiation compartment. Here the expression of K1 and K10 keratins increases, while that of K5 and K14 decreases.¹⁹ Cell-to-cell attachment increases dramatically in the stratum spinosum. Desmosomes develop in great numbers, in association with the rise in keratin intermediate filament bundles (Fig 4-7).

Adherens junctions for the attachment of actin microfilaments also increase in number. The juxtaposed cell membranes at the adherens junction are separated by a 20-nm cleft, slightly narrower than the 30-nm desmosomal cleft. In the adherens junction, the attachment plaques and the intercellular dense lines are not well defined. The molecular composition of desmosomes and adherens junctions is discussed in the section, "Desmosomes and adherens junctions," later in this chapter. Gap junctions are also present in high numbers between cells of this layer.

Membrane-coating granules (MCGs), or lamellar granules, are assembled in the Golgi complex (Fig 4-8). They contain lamellar plates of fatty acids, cholesterol, and sphingolipids. These lipid plates are re-

leased by exocytosis into the intercellular spaces at the upper layers of the stratum granulosum.

Stratum granulosum

The stratum granulosum derives its name from its content of keratohyalin granules (KHGs). Messenger ribonucleic acids (mRNAs) for filaggrin, the principal component of the KHG, and for loricrin and involucrin, precursors of the cell envelope, increase in amounts in the stratum granulosum. Synthesis of K1 and K10 declines in the stratum granulosum, as existing keratin filaments become increasingly stabilized by their association with KHG proteins. The K1 and K10 proteins are regarded as early markers, while filaggrin, loricrin, and involucrin are late markers of terminal keratinocyte differentiation. During the cornification process, profilaggrin is converted to filaggrin, a protein that associates with K1/K10 filaments to form a stabilizing interfilamentous matrix.

Membrane-coating granules continue to increase in number and migrate to the peripheral cytoplasm close to the plasma membrane in the outer layers of

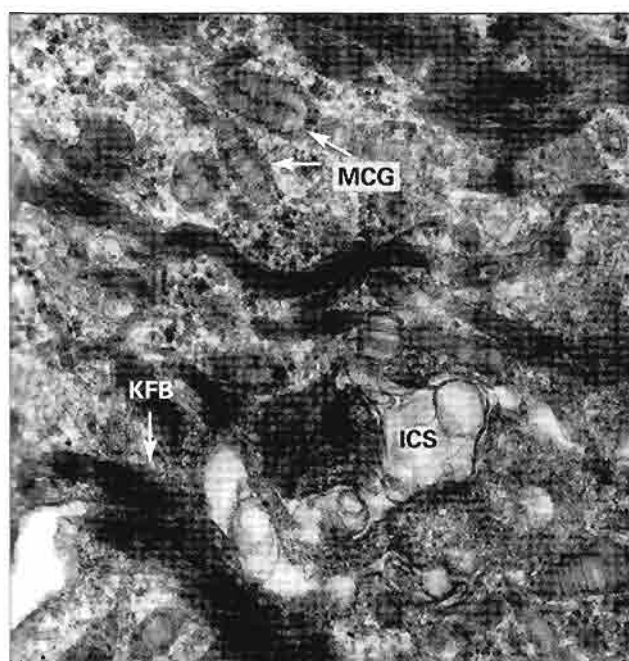


Fig 4-8a Electron micrograph of part of a cell in the stratum granulosum of hard palatal epithelium depicting numerous membrane-coating granules (MCG) and keratin filament bundles (KFB). (ICS) Intercellular space. (Original magnification $\times 34,000$.)

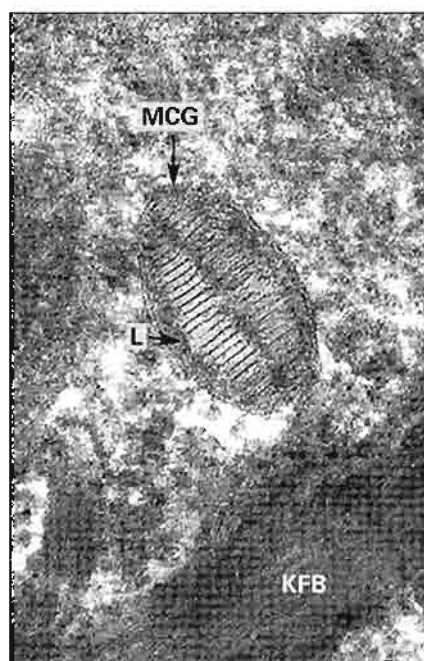


Fig 4-8b Higher magnification reveals the regular arrangement of the lamellar lipid plates (L) in the membrane-coating granules (MCG). (KFB) Keratin filament bundles. (Original magnification $\times 86,000$.)

the stratum granulosum (see Fig 4-8). Subsequent fusion of the MCGs with the plasma membrane releases lamellar plates of nonpolar lipids into the intercellular space to form a permeability barrier to water and water-soluble substances.^{20,21} The secretion of the contents of the MCGs coincides with the formation of the cell envelope (CE). Both events appear to be triggered by an increase in the cytosolic concentration of ionic calcium.²² New and convincing evidence for a claudin-based tight junctional network in the plasma membranes of the stratum granulosum of the epidermis has been published.²³ That these tight junctions participate in forming a barrier to water was shown in tracer studies and in the fact that claudin-deficient mice suffered rapid water loss and died at one day after birth.

With the perfection of confocal microscopy and the development of fluorescent antibodies to tight junction proteins, a zonula occludens was discovered at the junction between the stratum granulosum and the stratum corneum.²⁴ This tight junction participates in the formation of a permeability barrier to water.

The CE is a dense marginal band, roughly 15 nm thick, of highly insoluble proteinaceous material

formed by covalently cross-linked involucrin, loricrin, filaggrin, desmoplakin-like protein, and cysteine-rich protein (Fig 4-9).²⁵ Other CE proteins present in smaller amounts include small proline-rich proteins and cystatin.^{26,27} Involucrin protein serves as a scaffold for CE formation.^{25,28} Glutamine and lysine residues of involucrin provide cross-linkage sites for binding other CE proteins.²⁷

Keratinocyte transglutaminase (TGase-K) is responsible for catalyzing the cross-linking reaction.²⁹ To fabricate the CE, TGase-K must be anchored to the inner surface of the plasma membrane. An increase in cytosolic calcium ion concentration activates a phosphokinase-C signaling pathway, which in turn triggers the activation of TGase-K in upper-level cells.³⁰ Although mRNA for TGase-K is present in lower-level cells, the enzyme is expressed only in the stratum granulosum of cornifying epithelia and in the upper layers of noncornified oral mucosa.

Disulfide bonds introduced between cysteine-rich proteins also help to stabilize the CE structure. Keratin filaments are also anchored into the cell envelope by cross-linkage to various cell envelope proteins.^{25,31,32}



Fig 4-9 Electron micrograph of the cell envelope (CE), a thickened band of proteins fused to the inner surface of the plasma membrane of the cells of the stratum corneum. (ECS) Extracellular space; (Kf) keratin filaments. (Original magnification X 78,000.)

Biochemical analysis of CEs has shown that differences in composition exist among different epithelia. However, involucrin appears common to all CEs. The fact that the CE of cornified SSE is thicker than that formed in noncornified SSE suggests that there are differences in composition, and perhaps in construction, between the two.

The time taken for transition to stratum corneum varies; it is slower in skin than in oral mucosa. The stratum lucidum of skin represents layers of cells in transition to the stratum corneum. No stratum lucidum is present in oral cornified epithelium.

Stratum corneum

The outermost cells of cornified SSE consist of dead squames, which are shed from its surface. All organelles, including the nucleus, undergo autolysis during the conversion from the stratum granulosum. Stratum corneum chymotryptic enzyme, a serine protease whose expression is restricted to the outer-level cells of stratified squamous epithelia, may be involved in digestion of intercellular contacts in cells that undergo full cornification.³³ Stratum corneum chymotryptic enzyme is not found in normal noncornified oral mucosa.

The cornified squames are composed entirely of keratin filaments stabilized in an interfibrillar matrix. Filaments remain anchored to the plasma membrane via the cell envelope.³² Layer upon layer of

highly nondegradable cornified squames provides an efficient, renewable protective surface.

Recent evidence indicates that terminal differentiation (cornification) of keratinocytes shares many aspects of programmed cell death or apoptosis.^{18,34} Both processes involve endonuclease fragmentation of DNA, the loss of *Bcl2* expression, and the activation of transglutaminase (see chapter 13). The *Bcl2* gene is an inhibitor of apoptosis active in basal keratinocytes.¹⁸

Differentiation of the Oral Mucosa

The site variability of oral mucosal differentiation is readily apparent at the histologic and ultrastructural level. The microanatomic variation of oral mucosal structure has been described in detailed morphometric studies conducted by Schroeder.⁴¹ Site variation also is observed in the distribution of resident nonkeratinocytes, such as Merkel cells, Langerhans cells, and melanocytes.

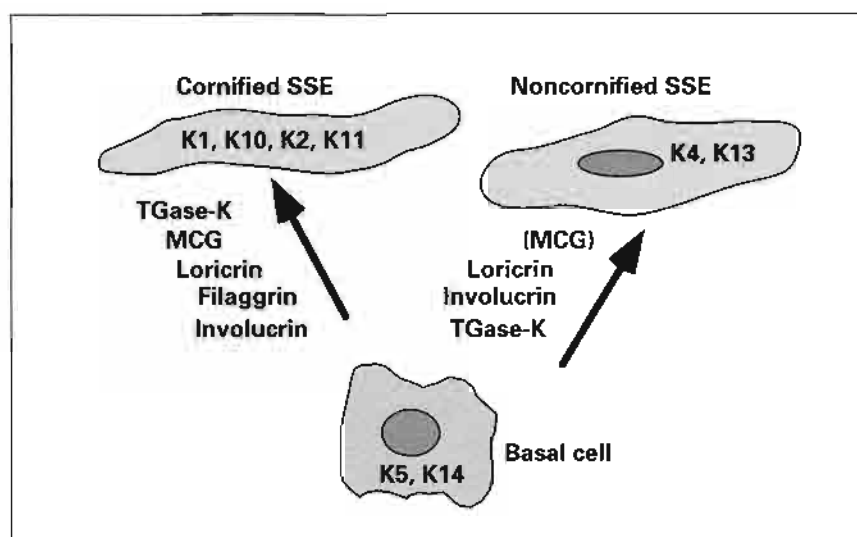
Differentiation patterns vary from fully cornified (epidermal-like) differentiation occurring on the hard palate (see Fig 4-11) to noncornified epithelium covering the buccal mucosa (see Fig 4-12).³⁵ Variations in thickness of differentiating and differentiated compartments vary from site to site, as well in ridges and in areas located over connective tissue papillae. Surface cells in fully cornified oral mucosa are arranged neatly, with closely approximated and slightly overlapping borders, while those of noncornified oral mucosa are arranged unevenly and are widely separated by intercellular clefts.³⁶

In general, cell renewal is faster in oral mucosa than in the epidermis. For example, in the mouse, the epithelium of the ventral surface of the tongue turns over four to five times faster than does the epithelium of the skin.

The expression of terminal differentiation products in noncornified SSE differs from that in cornified SSE (Fig 4-10). In noncornified SSE, the K4 and K13 keratins are expressed in suprabasal cells. Suprabasal cells in cornified SSE express high-molecular weight keratins (K1, K2, K10, and K11). Although loricrin, involucrin, and TGase-K are produced in upper-level cells of noncornified SSE, the available evidence suggests that the finished cell envelope is neither as thick nor as resistant as it is in cornified squames.

Membrane-coating granules in cornified SSE contain lamellar plates, while the smaller MCGs of non-

Fig 4-10 Expression of gene products during differentiation in cornified and noncornified stratified squamous epithelia (SSE). All basal cells express keratins K5 and K14. In suprabasal cells, K5 and K14 are downregulated; keratins K1, K2, K10, and K11 are expressed in cornifying cells, and keratins K4 and K13 are expressed in noncornifying cells, such as in the buccal mucosa. Filaggrin is expressed in association with high-molecular weight keratins. The membrane-coating granules (MCG) of noncornifying cells are different from the multilamellar types found in cornifying epithelia. (TGase-K) Keratinocyte transglutaminase.



cornified SSE contain a dense homogenous material devoid of lamellar plates. Studies of the permeability of tracer substances such as horseradish peroxidase indicate that a permeability barrier is present in noncornified oral mucosa at the level where the contents of the MCGs are secreted.³⁷ Differences in amounts, and in lipid composition, of the MCGs probably account for the greater permeability observed in noncornified SSE than in cornified SSE.

Another significant difference between the two is the absence of filaggrin expression in noncornified SSE, although small KHGs are sometimes present in upper-level cells.³⁸ The absence of the bundling protein, filaggrin, results in loosely organized filament networks in upper-level cells of noncornified epithelia. Differences in the amino acid composition of the terminal segments of the K4 and K13 keratin chains might also be a factor in the dispersed state of the filaments.

The normal pattern of keratin expression is altered in hyperplastic and cancerous lesions of the oral mucosa. The appearance of K8, K18, and K19, typical of simple highly proliferative epithelial cells, becomes prominent in oral leukoplakia and stratified squamous carcinoma.³⁹

The genetic and biochemical basis of microanatomic variation will become increasingly evident as advances continue to be made in the molecular biology of keratinocyte function. For a comprehensive description of cell differentiation in the hard palate, buccal mucosa, dorsum of the tongue, and floor of the mouth, the reader should consult Schroeder's extensive treatment of the subject.⁴¹

Hard palate

Cell differentiation in the hard palate is closely similar to that in the epidermis (Fig 4-11). Keratinocytes double their volume during conversion from the stratum basale to the stratum granulosum. Keratin types K1/K10, membrane-coating granules, and keratohyalin granules, all morphologic markers of fully cornified epithelium, are highly developed in the stratum granulosum. Keratinocytes in the upper layer (stratum corneum) produce a thickened cell envelope during the final stage of cornification. Quantitative comparison of epidermal and palatal stratum corneum has shown that fewer lipid lamellae are extruded from MCGs into the intercellular spaces of palatal epithelium, which correlates with the fact that palatal mucosa has relatively higher permeability than does skin.⁴⁰

Desmosomes occupy roughly two times more area on the surface of cells of the hard palate than do their counterparts in lining mucosa.⁴¹ The increased expression of desmosomal components may be a response to a need for greater structural integrity against forces generated during mastication. Adaptation to resist shearing forces is also evident in the epithelial-connective tissue interface, which is characterized by numerous lamina propria papillae (approximately 100/1.0 mm² of epithelial surface).

Although the cytoplasmic contents of the keratinocytes of skin and hard palate are generally similar, basal cells of the hard palate contain significantly more ribosomes and mitochondria than do their epi-

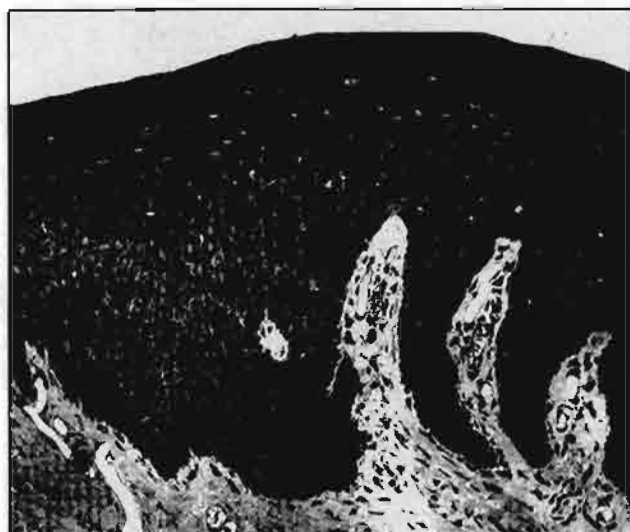


Fig 4-11 Light micrograph of a section of the hard palate. (Original magnification $\times 120$.)



Fig 4-12 Light micrograph of buccal mucosa depicting the stratum filamentosum (SF) and the stratum distendendum (SD). (CTP) Connective tissue papilla. (Original magnification $\times 120$.)

dermal counterparts.⁴¹ The ultrastructural appearance of the two tissues suggests that there is a difference in the content of the cornified squames. In cornified epidermal squames, keratin filaments appear less dense than the surrounding matrix; in cornified squames of the hard palate, keratin filaments cannot be distinguished from the surrounding homogenous matrix.⁴¹

Buccal mucosa

Unlike rigid cornified epithelia, buccal mucosa can undergo considerable distension—picture jazz trumpeter Louis Armstrong's greatly distended cheeks as he blew a high note. This property of distensibility is due to a lack of filaggrin protein and a different pattern of keratin expression in the keratinocytes (K4/K13 versus K1/K10) and to a greater amount of elastin in the submucosal connective tissue of the cheek.

Buccal epithelium represents the thickest region of oral SSE. Three strata characterize it: basale, filamentosum, and distendendum (Fig 4-12).⁴¹ There are approximately 70 connective tissue papillae per 1.0 mm² of mucosal surface. The epithelium lying over the tip of each connective tissue papilla is relatively thin (less than 0.2 mm).

Basal cells are comparatively small and have a high content of mitochondria and free ribosomes.

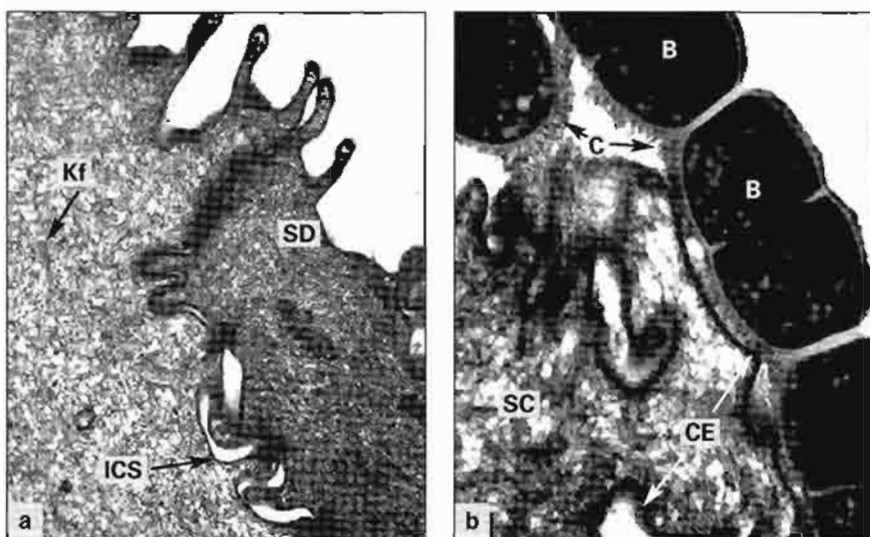
Keratin filaments make up about 20% of the cytoplasmic volume. Transit-amplifying cells entering the differentiation compartment form the stratum filamentosum. Differentiating cells increase in size and accumulate large numbers of keratin filaments. The Golgi apparatus and the cisternae of the RER account for a small component of the cytoplasm, a reflection of the fact that the bulk of protein synthesis is intracellular keratin. The existing RER may be engaged in the synthesis of proteoglycans and/or the lipids of the MCGs. Membrane-coating granules with an amorphous dense core and no lamellar plates develop in the stratum filamentosum and are concentrated beneath the plasma membrane along the junction of the stratum filamentosum and the stratum distendendum. Keratohyalin granules are not a normal component of buccal epithelium.

The cells of the stratum distendendum are generally flattened, with their long axis parallel to the mucosal surface. Mitochondria and ribosomes are greatly reduced in numbers. There are more glycogen particles, accounting for about 4% of the cytoplasmic volume.

Keratin filaments, consisting of K4 and K13 chains, form a loosely organized network taking up nearly 70% of the total cytoplasmic volume. The keratin filaments, 8 to 9 nm in diameter, are not bundled, as they are in highly cornified epithelia. This is probably because of the lack of filaggrin and the expression of a

Fig 4-13a Light micrograph of outermost cells of the stratum distendens (SD) of the buccal mucosa containing a loose network of unbundled keratin filaments (Kf). Cells are interdigitated along the intercellular spaces (ICS). (Original magnification $\times 60,000$.)

Fig 4-13b Bacteria (B) are commonly found attached to the surface cells (SC) of oral mucosa via capsular (C) components. (CE) Cell envelope. (Original magnification $\times 83,000$.)



different set of keratin molecules. Recent analysis of keratin gene expression in human buccal mucosa revealed that mRNAs for K1 and K10 are present in basal and lower-level cells but that K1 and K10 proteins are present in very small amounts.⁴² It was suggested that the K1 and K10 proteins are regulated at the posttranscriptional level and that their expression increases in pathologically altered buccal mucosa.

The cell envelope is generally thinner than in cornified SSE (Fig 4-13). Cell-cell interdigitation of microvillus-like processes and microridges are a characteristic surface feature of the outer-level cells of buccal mucosa (see Fig 4-13).⁴³ These structures increase the surface area for desmosomal contacts and increase the epithelial resistance to external forces. Bacteria are routinely found attached to the outer cells of both lining and fully cornified oral mucosa.

In general, there are fewer desmosomes in the keratinocytes of noncornifying SSE than in those of cornifying SSE. The smaller number of desmosomes in noncornifying epithelia reflects the lesser amount of keratin filament bundling (tonofilament formation) observed in those cell types.

Langerhans cells are found in clusters within the epithelium, around the connective tissue papillae, in numbers equal to those found in skin.⁴⁴ The concentration of Langerhans cells in the mouth is reported to vary inversely with the degree of keratinization.⁴⁴

Gingiva

The gingival epithelium is relatively thick and well cornified on its oral surface but thin and noncornified as it is reflected back to form the lining of the gingival sulcus and the junctional epithelial attachment (Fig 4-14).

Oral gingival epithelium

The OGE is cornified, impermeable to water-soluble substances, and attached firmly to a base of dense gingival connective tissue (Fig 4-15). Four clearly defined cell layers are present: the basal cell layer, the spinous cell layer, the granular cell layer, and the cornified cell layer. The basal cells make up the proliferation compartment of the epithelium, and the remaining layers form the differentiation compartment.

There is a high degree of interdigitation (rete peg formation) between the OGE and the underlying connective tissue. Contact between the two tissues is amplified by the presence of numerous serrated keratinocytes with prominent cell processes (pedicles) that protrude into the connective tissue. Basal cells attach to the lamina densa of the basal lamina through the formation of many hemidesmosomes. Anchoring fibrils made of type VII collagen bind the lamina densa to type I and type III collagen fibrils.

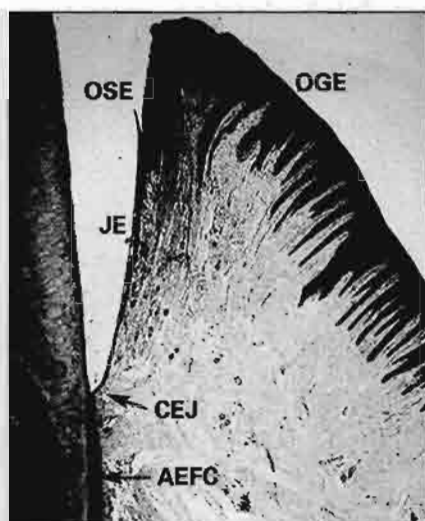


Fig 4-14 Light micrograph of gingival epithelium. (AEFC) acellular extrinsic fiber cementum; (CEJ) cemento-enamel junction; (D) dentin; (JE) junctional epithelium; (OGE) oral gingival epithelium; (OSE) oral sulcular epithelium. (Hematoxylin-eosin stain. Original magnification $\times 30$.)

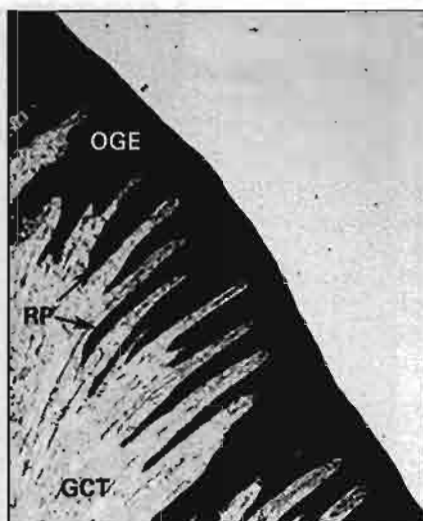


Fig 4-15 Light micrograph of oral gingival epithelium (OGE) and gingival connective tissue (GCT). Note the many rete pegs (RP). (Hematoxylin-eosin stain. Original magnification $\times 50$.)

Spinous layer cells of the OGE are specialized for cell-to-cell contact via their many desmosomes. These cells contain many keratin filament bundles (tonofibrils) that associate peripherally with the attachment plaques of desmosomes. The number of desmosomes per cell doubles from the basal layer to the spinous layer. Cell contacts of the gap junction variety are also abundant. Nonkeratinocytes located in the OGE include melanocytes, Langerhans cells, and Merkel cells.

Similar to the differentiation pattern found in skin, the stratum granulosum of the OGE contains membrane-coating granules, keratohyalin granules, and numerous tonofibrils. The transition to the stratum corneum is abrupt. The flattened cornified squames form a relatively thick protective covering over the connective tissue and the epithelial attachment.

Parakeratinization, a condition characterized by incomplete disintegration of the nucleus and some cytoplasmic organelles, is usually observed in the stratum corneum of the OGE. Complete digestion of the nucleus and organelles, accompanied by a more complete and uniform cornification as found in skin, is called *orthokeratinization*.

Oral sulcular epithelium

The oral sulcular epithelium (OSE) extends apically from the crest of the marginal gingiva to the junc-

tional epithelium (JE) (see Fig 4-14). Deep interdigitations between basal cell podocytes and connective tissue are not as evident beneath the OSE. Although the OSE is stratified, it does not contain a clearly defined stratum granulosum, nor does it normally undergo cornification.

The differentiating compartment contains inner and outer zones. The inner zone resembles a spinous layer, but individual cells contain fewer tonofibrils and desmosomes than do cells in the spinous layer of the OGE. The cells of the inner zone tend to be flattened and to lie parallel to the epithelial surface. The outer zone contains viable cells with intact nuclei and abundant cytoplasmic organelles. The most superficial cells, which are shed into the sulcus, demonstrate considerable variation in shape and density; some are thin and darkly basophilic, while others are large and lightly stained. Different degrees of hydration and plasma membrane integrity may account for these differences.

Keratohyalin granules and MCGs are rarely observed in cells of the OSE. The outermost cells contain a moderate amount of RER, Golgi membranes, and dense granules. Although the nature of the dense granules has not been satisfactorily established, some reports suggest that they belong to the lysosomal system, while other reports have indicated that they might be a variant of the MCG.

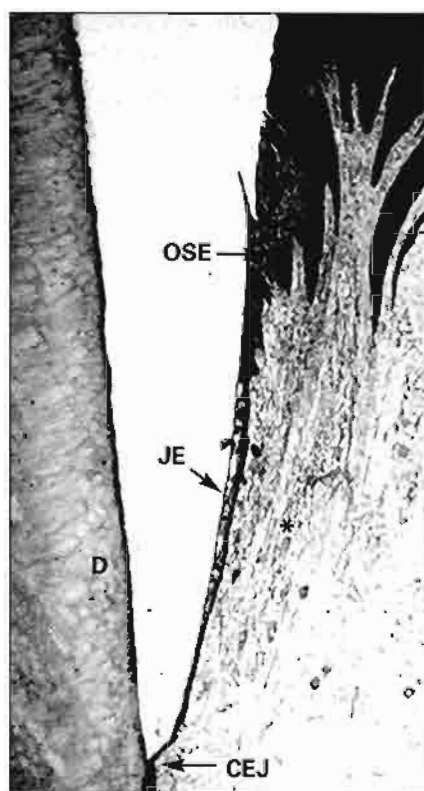


Fig 4-16 Light micrograph of the junctional epithelium (JE) and the oral sulcular epithelium (OSE) bordering the enamel. (CEJ) Cementoenamel junction; (D) dentin; (*) gingival connective tissue. (Hematoxylin-eosin stain. Original magnification $\times 30$.)

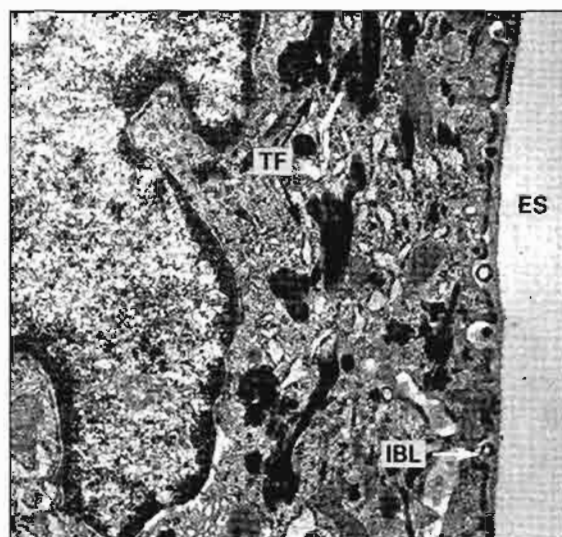


Fig 4-17 Transmission electron micrograph of the attachment of the outermost junctional epithelium cell to the inner basal lamina (IBL). (ES) Enamel space; (TF) tonofilaments. (Original magnification $\times 5,000$.)

Junctional epithelium

Junctional epithelium maintains a direct attachment to the tooth surface. The basal cells of the JE are separated from the connective tissue by the external basal lamina. The interface between the JE and the underlying connective tissue is relatively smooth (Fig 4-16), unlike the condition found in the OGE. Epithelial rete peg formation from the JE (and the OSE) is a condition found in highly inflamed connective tissue.

Although JE does not exhibit true phenotypic stratification, the outermost cells tend to be elongated and to lie with their long axis parallel to the tooth surface. Suprabasal cells of the JE express markers typically found in basal cells and simple epithelia. The JE tapers from its coronal end, which may be 10 to 20 cells wide, to its apical termination, which is a few cells wide and located at the cemento-enamel junction in healthy tissue (see Fig 4-16).

Epithelial cells of the JE in contact with the tooth surface also produce an internal basal lamina and

are anchored to this basal lamina by numerous hemidesmosomes. Of interest is the observation that keratin tonofilaments are not inserted into the hemidesmosomes along the internal basal lamina (Fig 4-17). The internal basal lamina is approximately three times thicker than the external basal lamina. It contains laminin and proteoglycans. Cells in contact with the internal basal lamina express the $\alpha 6 \beta 4$ integrin, a laminin receptor. The cells in contact with the internal basal lamina contain a relatively well-developed RER and numerous Golgi components.

Electron microscopic cytochemical studies have shown that the cells of the JE contain a moderately well-developed lysosomal system and participate in the phagocytosis of material from the intercellular space. Junctional epithelium cells show no signs of synthesis of MCGs, a finding that agrees with the fact that the JE is highly permeable to water-soluble substances. The chief barrier to passage of substances larger than 100 kDa is provided by the external basal lamina.

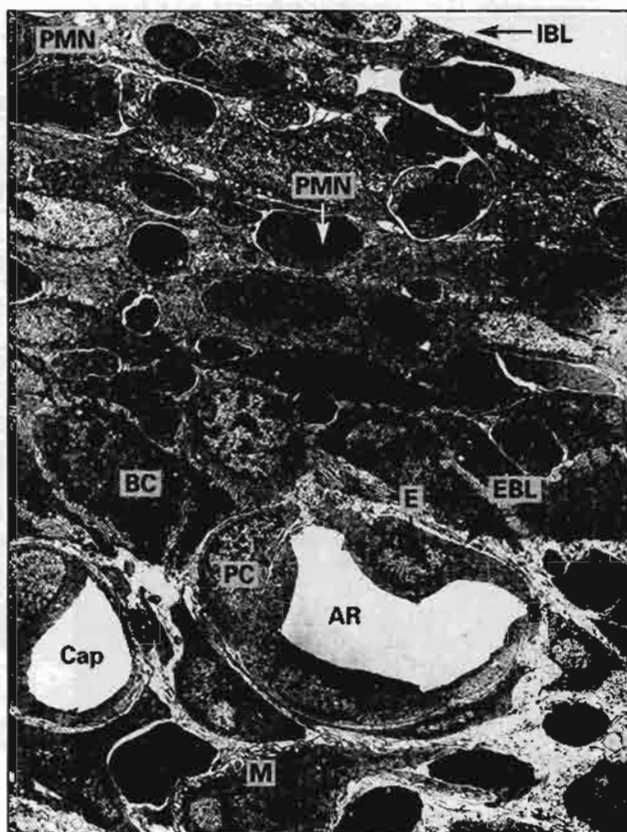


Fig 4-18 Transmission electron micrograph of polymorphonuclear neutrophil (PMN) migration through the junctional epithelium. (AR) Arteriole; (BC) basal cell; (Cap) capillary; (E) endothelium; (EBL) external basal lamina; (IBL) internal basal lamina; (M) macrophage; (PC) pericyte. (Original magnification $\times 1,800$.)

Because of the absence of an effective permeability barrier between the cells of the JE, it provides an open pathway for the penetration of bacterial antigens, lipopolysaccharides, and enzymes from the sulcus to the connective tissue. Sulcular fluid, a protein-rich fluid derived from transudation of serum and extracellular fluid, flows in an outward direction through the JE. It contains antibodies, complement, and enzymes that form an antibacterial defense system. The JE also serves as the major pathway for the transmigration of neutrophils into the gingival sulcus (Fig 4-18).

Although the JE contains fewer numbers of cell-to-cell junctions per equivalent length of plasma membrane than does the OGE, there are many well-developed gap junctions and desmosomes. Small adherens junctions are also present. In general, there appears to be an inverse relationship between the numbers of infiltrating polymorphonuclear neutrophils and epithelial cell-to-cell junctions.

In healthy teeth, which have not had any prior loss of attachment, the JE (epithelial attachment) ends at the cemento-enamel junction (see Fig 4-16). Densely packed collagen bundles are anchored to the acellular extrinsic fiber cementum just below the terminal point of the JE. These collagen bundles form the connective tissue attachment. The stability of this connective tissue attachment is a key factor in limiting the migration of the JE. In gingivitis and periodontitis, resorption of collagen along the root surface beneath the JE removes a major barrier to epithelial migration.

Mucosa of the tongue

The mucosa of the dorsal surface of the tongue contains a mix of cornified and noncornified SSE, while that over the lateral and ventral surfaces is a non-cornified lining SSE. Three types of papillae characterize the dorsum of the tongue: filiform, fungiform, and circumvallate (Figs 4-19 to 4-21). A fourth type, the foliate papillae, is located along the lateral and posterior borders of the tongue. Each type is differentiated to perform a specialized function while maintaining its primary role as a protective covering for the internal tissue components. All papillae contain a highly vascularized central connective tissue core (dermal papilla). The three-dimensional architectural pattern of the papillary connective tissue cores and their terminal vascular beds have been displayed in dramatic fashion in scanning electron micrographs of vascular corrosion casts⁴⁵ (Fig 4-22).

Filiform papillae are the smallest, yet most abundant, structural specializations of the dorsum of the tongue. They have a modified conical shape characterized by curvature toward the back of the tongue. The demarcation between the anterior and posterior compartments of the filiform papilla of the mouse is rather sharply defined (Figs 4-23 and 4-39).

The anterior compartment and the interpapillary SSE contain K4/K13 keratin chains similar to those found in esophageal and oral lining epithelia. The anterior compartment also contains K6/K16 and trichohyalin protein, a member of the filaggrin family of filament-bundling proteins. Trichohyalin is characteristically present in the "soft" compartments of hair follicles in association with K6/K16 filaments.⁴⁶ In the cells of the stratum granulosum of the anterior compartment, the trichohyalin is colocalized with filaggrin (see Fig 4-23). In the cornification process, trichohyalin and filaggrin are dispersed in the cytoplasm in association with keratin filaments.

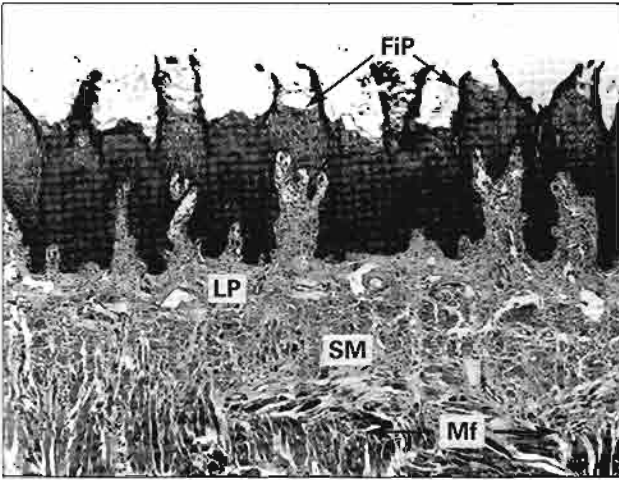


Fig 4-19 Light micrograph of filiform papillae (FiP). (LP) Lamina propria; (SM) submucosa; (Mf) muscle fibers. (Original magnification $\times 100$.)

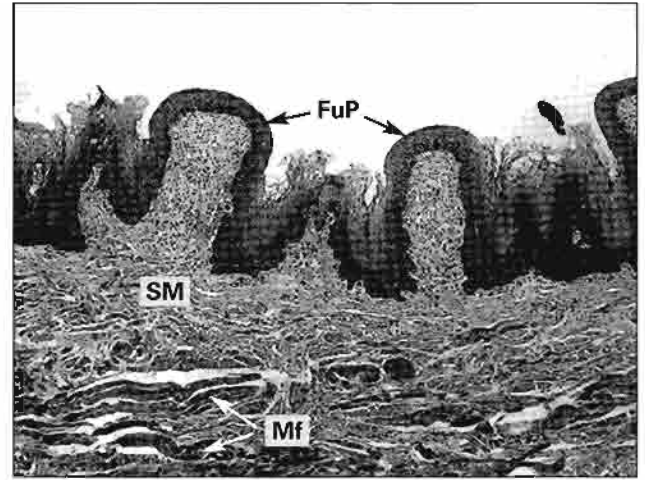


Fig 4-20 Light micrograph of fungiform papillae (FuP). (SM) Submucosa; (Mf) muscle fibers. (Original magnification $\times 100$.)

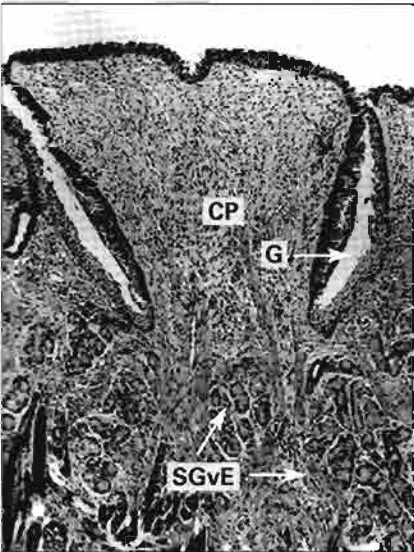
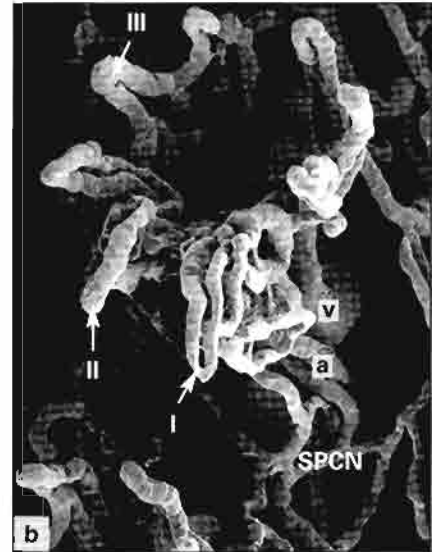


Fig 4-21 Light micrograph of circumvallate papilla (CP) surrounded by the circumvallate groove (G). Salivary glands of von Ebner (SGvE) are present in the underlying connective tissue. (Original magnification $\times 100$.)



Figs 4-22a and 4-22b Scanning electron micrographs of the dorsal vascular bed of the tongue. (a) (FP) Filiform papillae; (SPCN) subpapillary capillary network. (Original magnification $\times 6$.) (b) Terminal capillary loops (I, II, and III) of the filiform papillae project upward from the underlying subpapillary capillary network (SPCN). (a) Arteriole; (v) venule. (Original magnification $\times 22$.) (Adapted from Kishi et al,⁴⁵ with permission from John Wiley & Sons.)



In the posterior compartment, the K1/K10 pair of keratins is expressed without KHGs (see Fig 4-23). Trichohyalin and filaggrin are not found in the stratum granulosum cells of the posterior compartment. This epidermal, or "hard," differentiation pathway provides a more rigid product than does the "soft" esophageal phenotype, causing the filiform papilla to curve posteriorly.

Analysis of cell turnover and the expression of genetic markers in mice indicates that the filiform papilla

is a polyclonal structure. The border between the anterior and posterior compartments demarcates the separation between two adjacent cell clones, each with different keratin and keratin-associated proteins.

A similar distribution pattern of keratins, trichohyalin, and filaggrin has been demonstrated in the filiform papilla of humans. The larger human filiform papilla encompasses many epidermal proliferation units that differentiate along esophageal and

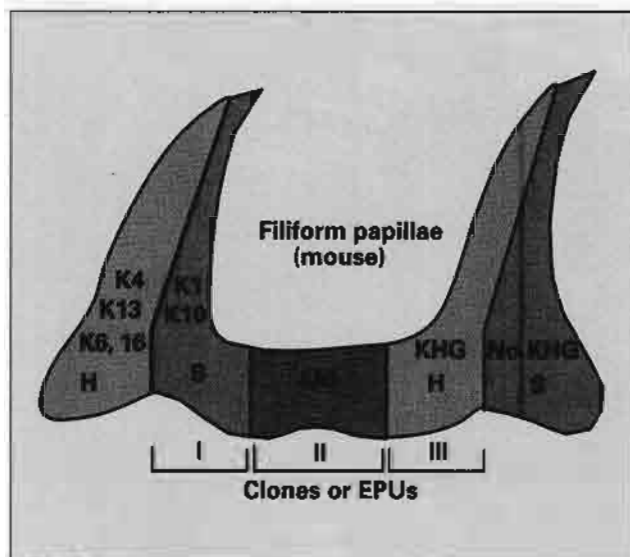


Fig 4-23 Clonal differentiation in mouse filiform papillae. In the anterior compartment or epidermal proliferation unit (EPU), cell differentiation follows a hairlike pattern involving the expression of keratin (K) types 4, 6, 13, and 16, as well as keratohyalin granule (KHG) proteins. A skinlike type of differentiation is present in the posterior compartment, including expression of K1 and K10 but not the presence of KHGs. The segment of mucosa between adjacent papillae resembles lining or buccal mucosa. (H) Hairlike; (S) skinlike; (LM) lining mucosa-like. (Adapted from data presented in Manabe and O'Guin,⁴⁶ with permission from Blackwell Publishing.)

epidermal pathways. The resulting appendage has several cornified spikes that have a slight anterior-to-posterior slant. In some animals, the posterior compartments produce barblike points that give the dorsal surface of the tongue a rasplike texture. In addition to helping stabilize food particles during mastication and swallowing, filiform papillae serve in the tongue's cleansing action on oral mucosa and tooth surfaces. Filiform papillae are especially well developed in animals that use the tongue to groom body hair.

Fungiform papillae are dome-shaped appendages covered by a thin cornified SSE (see Fig 4-20). Terminal differentiation products of the epithelium include K1/K10 keratins and filaggrin. Fungiform papillae are concentrated in the anterior third of the tongue, serving as specialized touch and taste organs. Most fungiform papillae contain taste buds in the apical epithelium. The underlying connective tissue papillae is richly innervated and well vascularized. Encapsulated nerve endings resembling Meissner's corpuscles of skin are located in the most apical parts of the connective tissue papillae.

The circumvallate papillae are the largest of the four types. They are about 2.5 mm wide and 1.0 mm deep. These are not true papillae; they do not project above the surface of the tongue but rather are defined by an invagination of the epithelial surface (Figs 4-21 and 4-24). They occur at the junction between the anterior two thirds and the posterior one third of the tongue in a single V-shaped row, whose

apex points anteriorly. A cornified SSE covers the apical and lateral surfaces of the circumvallate papillae. The lateral surfaces, bordering the trench formed by the invagination of the epithelium, contain hundreds of taste buds. A smaller number of taste buds are present in the apical epithelial surface. The connective tissue is richly vascularized and innervated. The circumvallate trench or groove is supplied with the secretions of the salivary glands of von Ebner.

Foliate papillae are located at the posterior lateral borders of the tongue. They consist of a series of ridgelike structures formed by mucosal folds oriented in a dorsoventral direction. The overlying epithelium is a cornified SSE like that of the dorsum of the tongue. Numerous taste buds are present in the lateral walls that separate the adjacent papillae.⁴⁷

Floor of the mouth

Cell strata and cell differentiation in the floor of the mouth resemble the pattern described for buccal mucosa, yet the epithelium is only half as thick as that of buccal mucosa epithelium. This portion of the oral mucosa demonstrates the highest permeability to water-soluble substances and is a preferred site for rapid transmucosal absorption of medication.⁴⁸ Wide epithelial ridges and sparsely distributed, short, fingerlike, connective tissue papillae characterize the epithelium-connective tissue interface.

Fig 4-24a Taste buds (TB) are located in the lateral epithelium of the circumvallate papilla (CP). (Original magnification $\times 160$.)

Fig 4-24b At high magnification, the taste bud (TB) is observed to communicate with the circumvallate groove via a taste pore (TP). (Original magnification $\times 250$.)



Basic Science Correlations

Keratin structure

Keratin intermediate filaments are essential to the mechanical strength of the stratified squamous epithelia.⁴⁹ Keratins are coded by 30 different genes. Keratin proteins occur in two major forms: acidic proteins (type I) and neutral-basic proteins (type II). In general, epithelial cells express keratins in pairs, a type I molecule along with its type II counterpart.

All keratin molecules contain a similar central core shared by all members of the family of intermediate filament proteins.⁵⁰ The core is a highly conserved region of the molecule made up of four α helical segments (1A, 1B, 2A, and 2B) separated by three non-helical linker sequences (L1, L2, and L3) (Fig 4-25).

Diversity among keratin filaments resides in non-helical extensions at the amino and carboxy terminals (H, V, and E end domains). The H domains are high-homology segments that are important in filament assembly. The V domains represent the most variable segments. Charged terminal amino acid sequences constitute the E domains. The V and E domains protrude from the surface of the completed filament and account for the functional diversity of the various types of filaments.

Filament assembly begins by parallel association of a type I chain with its type II counterpart. The paired molecules are positioned in close register, permitting maximum interchain hydrophobic interac-

tion, resulting in the formation of a coiled-coil dimer (Fig 4-26). Two dimers align in antiparallel fashion, either in register or in a staggered relationship, to form tetramers, the insoluble building blocks of the protofilament. The H1 subdomains of the type II chains play a direct role in keratin filament assembly, including stabilization of the tetramer molecule.⁵¹ Protofilaments aggregate in a lateral, staggered association to form 10-nm filaments.

In cross section, mature keratin filaments contain 12 to 20 dimers. Keratin filaments are subsequently bundled and assembled into macromolecular networks that radiate throughout the cytoplasm. Large filament bundles, the tonofilaments of light microscopy, are anchored in desmosomal and hemidesmosomal attachment plaques, providing a cytoskeletal fabric to stabilize the epithelium on a cellular and tissue level. The molecular connection between keratin filaments and desmosomes occurs between the amino terminal "head" of type II epidermal keratins (K1, K2, K5, and K6) and the carboxy terminal "tail" domains of desmoplakin 1, a component of the desmosome attachment plaque⁵² (see "Desmosomes and adherens junctions" and "Basal attachment apparatus," later in this chapter).

Recent studies have shown that intermediate filament systems are in dynamic equilibrium with a pool of soluble precursors, probably made up of dimers. Although the exact mechanisms that regulate the assembly of filament systems and their association with other components of the cell are unknown, there is

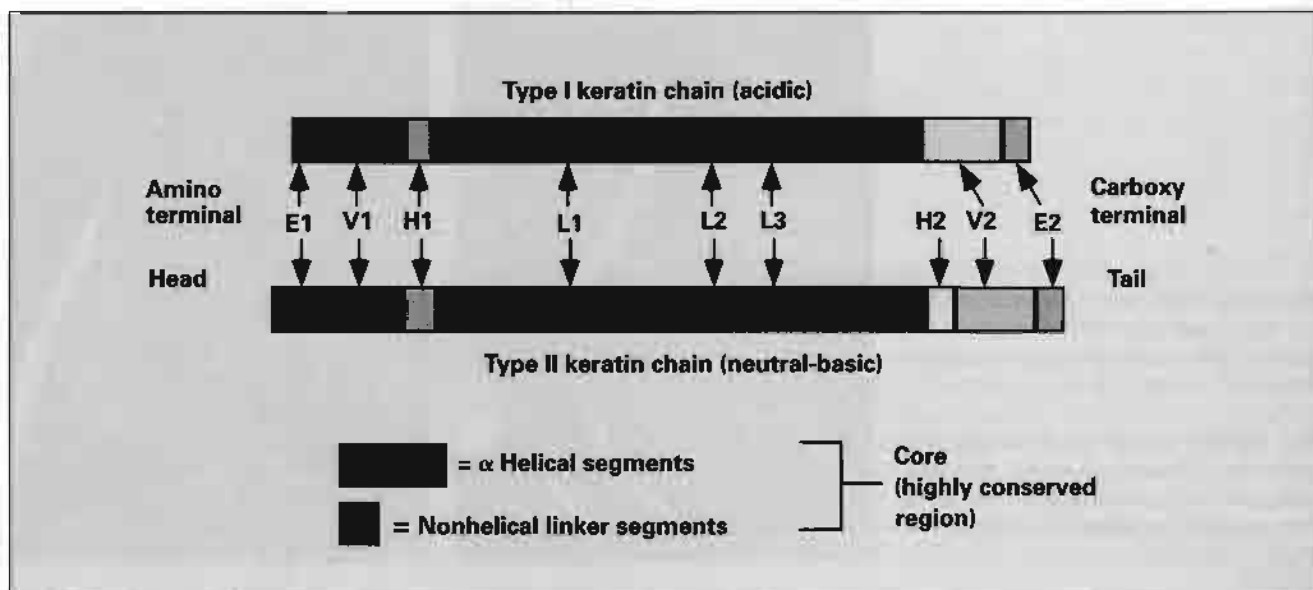


Fig 4-25 Basic molecular structure of type I and type II keratin polypeptide chains. Each chain has a large, highly conserved region made up of α helical segments (1A, 1B, 2A, and 2B) separated by short, nonhelical linker sequences (L1 to L3). Variable segments at the amino terminal (V1) and carboxy terminal (V2) and charged end groups (E1 and E2) provide for chain diversity and functional differences among the various genetically different keratins. Phosphorylation of the end domains is believed to control assembly and disassembly of filament bundles and interaction with desmosomal attachment proteins. (H1, H2) High homology domains.

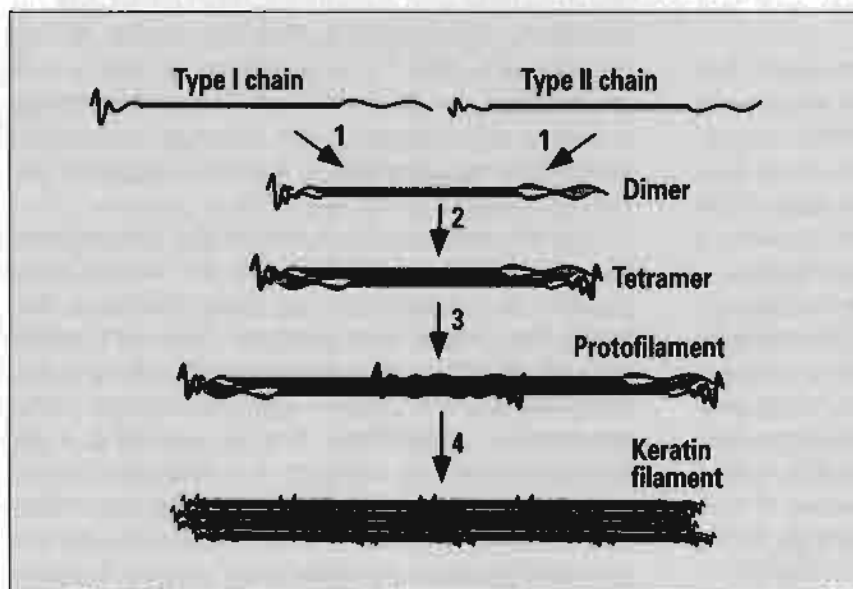


Fig 4-26 Keratin filament assembly. Keratin filaments are formed from equal numbers of type I and type II chains by the assembly of dimers, tetramers, and protofilaments. Dimers are stabilized by inter-chain hydrophobic bonds. Tetramers result from antiparallel association of dimers. Protofilaments are staggered during lateral aggregation to form the 10-nm keratin filament visible in the electron microscope.

growing evidence that phosphorylation of serine residues on the end domains of the keratin chains is a required step.⁵³

Intact keratin bundles are essential for maintaining the integrity of cells against mechanical stress. Mutations in the genes encoding keratin lead to frag-

ile keratinocytes.^{54,55} Patients who inherit these disorders develop extensive blisters, especially in areas of skin and mucosa that are exposed to high mechanical stress (see "Disorders of epithelial attachment," later in this chapter).

Control of keratinocyte differentiation

Numerous factors control keratinocyte differentiation (Fig 4-27). Active metabolites of vitamin D₃ act in an autocrine pathway to decrease keratinocyte proliferation and to increase cell differentiation.⁵⁶ Vitamin D₃ activates a vitamin D response element controlling the gene for phospholipase C- γ , thereby increasing the level of this enzyme.⁵⁷ When phospholipase C- γ is activated, it generates inositol triphosphate and diacylglycerol from phosphoinositol biphosphate. Inositol triphosphate opens calcium channels in the membranes of elements of the smooth endoplasmic reticulum, releasing calcium ions into the cytoplasm. Calcium acts as a second messenger to increase the permeability of plasma membrane cation channels. As more calcium enters the cell from the extracellular milieu, keratinocyte differentiation is stimulated. Activation of protein kinase C by diacylglycerol also plays a significant role in the vitamin D₃ response by stimulating the assembly of epithelial adherens junctions.⁵⁸

Epidermal growth factor (EGF) and transforming growth factor α (TGF- α) exert a mitogenic effect on basal cells via interaction with EGF receptors. The receptor for EGF has been localized in basal cells of the oral mucosa.^{59,60} Heparin-binding epidermal growth factor-like growth factor (HB-EGF), a new member of the EGF family of proteins, has been shown to have a positive effect on keratinocyte proliferation.⁶¹

Transmembrane signaling by EGF, TGF- α , and HB-EGF occurs through the activation of tyrosine kinase sites on the cytoplasmic domain of the EGF receptors. Downstream signaling events include the formation of inositol triphosphate and the elevation of intracellular calcium.⁶² Under normal conditions, EGF signaling leads to increased levels of the apoptosis suppressor protein, bcl-xl, thereby increasing keratinocyte survival and growth.⁶³ Both TGF- α and HB-EGF also exert a paracrine growth-promoting action on dermal fibroblasts.

Inhibition of the tyrosine kinase activity on the EGF receptors abolishes cell proliferation while promoting the expression of differentiation markers K1 and K10.⁶⁴ It has also been reported that apoptosis of keratinocytes in vitro can be induced by blocking the action of EGF receptor.⁶⁵

Insulin-like growth factor 1, originating in connective tissue, appears to act as a paracrine stimulator of keratinocyte proliferation. Receptors for insulin-like growth factor 1 are expressed on epidermal basal cells but are downregulated on cells entering the differentiation compartment.⁶⁶ During wound healing,

	Proliferation	Differentiation
EGF	↑	
KGF	↑↑	
Retinoids	↑	↓
Ca ⁺⁺		↑
TGF- α	↑	
TGF- β	↓	↑
α/β Integrins + ligand		↓

Fig 4-27 Some factors that influence growth and differentiation of the stratified squamous epithelium. Upregulation (green arrows) and downregulation (red arrows) of genes that control cell division and terminal differentiation. (EGF) Epidermal growth factor; (KGF) keratinocyte growth factor; (TGF) transforming growth factor.

insulin-like growth factor 1 and HB-EGF are increased in wound fluid. The synergistic effect of these growth factors has been shown to increase epithelial cell proliferation 40- to 50-fold.⁶⁷

Keratinocyte growth factor (KGF), a member of the fibroblast growth factor family, is produced by lamina propria fibroblasts.⁶⁸ The receptor for KGF is expressed by oral mucosa keratinocytes.⁶⁹ Acting in a paracrine pathway, KGF exerts a powerful stimulus for epithelial cell proliferation.^{70,71} Interleukin 1 β and interleukin 6 are also able to increase keratinocyte proliferation by stimulating the production of KGF.⁶⁸ Both KGF and interleukin 6 are elevated during wound healing and in inflammation. The expression of KGF is suppressed by glucocorticoids.⁷² Hepatocyte growth factor/scatter factor is another paracrine factor originating in connective tissue that elicits keratinocyte proliferation and migration.⁷¹

Transforming growth factor β inhibits DNA synthesis in basal cells and promotes terminal differentiation. Transforming growth factor β is secreted by basal and suprabasal cells in a latent form. Activation must take place before it can interact with receptors on the keratinocyte plasma membrane. Transforming growth factor β appears to have a role in decreasing the division of basal cells and triggering terminal dif-

ferentiation of keratinocytes. The synthesis of type VII collagen and the formation of anchoring fibrils is stimulated by TGF- β .⁷³

It is a well-established fact that high levels of vitamin A cause normally cornified epithelia to undergo mucous metaplasia, a condition wherein cornified surface cells are replaced by noncornified cells typical of lining mucosa. In contrast, vitamin A deficiency can cause an opposite effect, ie, squamous metaplasia. In that condition, cells of noncornifying epithelia are induced to undergo cornification.⁷⁴

Recent *in vitro* studies indicate that vitamin A suppresses terminal differentiation of oral and skin SSE by decreasing the expression of keratins (K1, K10, K5, K14, K6, and K16), involucrin, and filaggrin.^{75,76} The keratins of simple epithelia, K8, K18, K13, and K19, are increased by vitamin A.⁷⁶

The site of action of vitamin A is believed to be at the gene transcription level.^{75,77} The active form of vitamin A, retinoic acid (RA), binds to several nuclear retinoic acid receptors (RARs). The RA-RAR complexes control the transcription of mRNAs for several keratinocyte differentiation products, such as K1 and filaggrin.⁷⁴ The early growth response gene 1 (*ERG1*), a transcription regulator, has been shown to increase *in vivo* in response to application of vitamin A to skin.⁷⁸ Cellular RA-binding proteins (CRABPs) regulate the level of RA available for interaction with RARs. Lamina propria fibroblasts are able to influence epithelial differentiation by producing soluble factors that control the activity of epithelial CRABPs, thereby affecting the availability of RA to bind to its nuclear RARs.⁷⁶

Calcium plays a key role in keratinocyte differentiation. While basal cells maintain a low calcium concentration, stratification and terminal differentiation require a gradual, controlled increase in cytosolic calcium in suprabasal cells. Desmosomal assembly, release of MCGs, and transglutaminase activity require an elevated intracellular calcium ion concentration. Calcium also stimulates the transcription of keratins K1 and K10 and loricrin.

Mediators of inflammation have direct effects on keratinocytes. Prostaglandin E stimulates proliferation of keratinocytes by activation of the cyclic adenosine monophosphate signaling pathway.⁷⁹ Interferon γ , a cytokine produced by activated lymphocytes, inhibits growth while stimulating terminal differentiation of keratinocytes.⁸⁰ Interferon γ upregulates matrix metalloproteinase 1 and stromelysin (matrix metalloproteinase 3) in cultured keratinocytes without affecting the level of matrix metalloproteinase inhibitor expression.⁸¹ Interferon γ also stim-

ulates the expression of intercellular adhesion molecule 1 and major histocompatibility complex II in keratinocytes, providing for leukocyte adhesion and potential antigen presentation to lymphocytes within the epidermis.^{82,83} Tumor necrosis factor downregulates production of bullous pemphigoid antigen 1 (BPAG-1), a component of the hemidesmosome.⁸⁴ Interleukin 1 promotes keratinocyte differentiation by increasing the expression of CRABP type II and the small, proline-rich protein component of the cell envelope.⁸⁵

Nerve growth factor participates in the autocrine stimulation of the growth and survival of keratinocytes, especially in periods of inflammation.⁸⁶ Local nerve fibers are sustained by the neurotrophic effect of nerve growth factor secreted by keratinocytes. Nerve growth factor exerts a protective action on melanocytes during high levels of ultraviolet radiation. The neuropeptide, substance P, has been shown to stimulate keratinocyte proliferation via a calcium second messenger response.⁸²

Parasympathetic and sympathetic neurotransmitters and their receptors are expressed by epidermal keratinocytes. The biologic responses to epinephrine, norepinephrine, and acetylcholine, achieved chiefly through modulation of the cytoplasmic calcium concentration, have been reviewed by Grando⁸⁷ and Schallreuter.⁸⁸

Relationship among integrin expression, transforming growth factor β , cell division, and cell differentiation

Expression of integrins is highest in the basal cell layer.⁸⁹ The integrin β 1 subunit participates in cell-to-matrix adhesion by forming the α 2 β 1 integrin receptors for collagen and laminins and the α 3 β 1 integrin receptors for laminin.⁹⁰ The α 6 β 4 integrin is a component of the hemidesmosome. It binds to the laminin 5 component of the anchoring filaments. The integrin α 6 β 4 is expressed only in basal cells and displayed solely on the basal cell membrane. In general, α 2 β 1 and α 3 β 1 integrins are expressed on basal cells and cells of the first few layers of the stratum spinosum, where they are localized at cell-to-cell adhesions.⁹¹

Stem cells have a high level of β 1 integrin expression, while transit-amplifying cells have a reduced expression of β 1.¹² *In vitro* studies indicate that when β 1 integrin receptors are occupied by ligands (coll-

gen, laminin, fibronectin, or anti- $\beta 1$ antibodies) keratinocyte differentiation is blocked. Analysis of $\beta 1$ integrins localized at cell-to-cell adhesions of keratinocytes in the differentiation compartment have shown that they are not in the ligand-occupied conformation and are thus unlikely to be involved in signal transduction.⁹² Thus the downregulation of $\beta 1$ integrin expression, leading to detachment of cells from the basal lamina, is viewed as a possible trigger of keratinocyte differentiation. Reduced expression of the hemidesmosome-specific $\alpha 6 \beta 4$ integrin would appear to be necessary as well.

On the other hand, overexpression of $\beta 1$ integrins in suprabasal cells leads to hyperproliferation of epithelia.⁹³ In psoriasis, a skin condition characterized by accelerated turnover of epithelial cells, the $\alpha 5 \beta 1$ fibronectin receptor is increased.⁹⁴ Increased expression of $\beta 1$ integrins is beneficial when accelerated proliferation of epithelial cells is needed to repair a wound. In wound epithelium, the $\alpha 5 \beta 1$ fibronectin receptor is increased, thereby promoting cell division and migration.⁹⁵ Undoubtedly many factors acting on several signaling pathways are involved in the control of stem cell division and keratinocyte differentiation.

One growth factor that has received considerable attention in epithelial proliferation is TGF- β . Transforming growth factor β plays a key role in keeping cells in a nonproliferation state.⁹⁶ Basal layer epithelial cells remain in the G1 phase of the cell cycle while under the influence of TGF- β . The regulatory role of TGF- β in controlling cell proliferation is believed to be due to its ability to increase the expression of the cyclin-dependent kinase inhibitors, such as proteins p27 and p15 (see chapter 12).⁹⁷ Transforming growth factor β also stimulates the expression of K5 and K14, the keratins of the basal cell layer.⁹⁸ In response to wounding, TGF- β activates the expression of $\alpha 5 \beta 1$, $\alpha \nu \beta 6$, and $\alpha \nu \beta 5$, a set of integrins not normally used by keratinocytes, but needed for keratinocyte migration over the wound bed.⁹⁹

Secreted by keratinocytes, TGF- β acts as an autocrine factor to regulate the expression of integrins and extracellular matrix components. The synthesis of integrins and basement membrane components is stimulated by TGF- β , thereby maintaining the attached, nondifferentiated state (Fig 4-28).¹⁰⁰ In a negative-feedback loop, contact with the basement membrane, involving integrin-extracellular matrix interaction, downregulates the expression of TGF- β and ultimately the further expression of integrins and basement membrane components (see Fig 4-28). Thus, as integrin expression is reduced, the cells

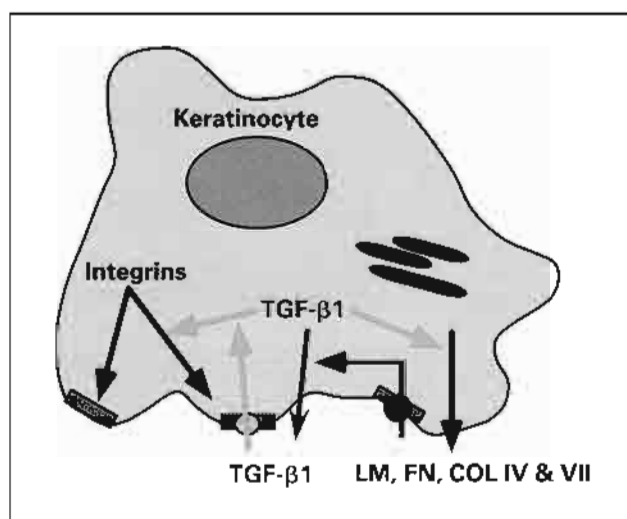


Fig 4-28 Interaction among the expression of transforming growth factor $\beta 1$ (TGF- $\beta 1$), basement membrane components, and integrins. TGF- $\beta 1$, secreted by keratinocytes (black arrows), acting as an autocrine, binds to its receptor (green circle), upregulating the expression of integrins and the secretion of basement membrane components (green arrows). Increased integrin binding (especially the $\beta 1$ subunit) to basement membrane ligands such as laminin (LM), fibronectin (FN), and collagen (COL) type IV (red circle) leads to negative-feedback downregulation of the expression of TGF- $\beta 1$ (red arrow).

have a greater tendency to detach from the substratum and undergo terminal differentiation.

Desmosomes and adherens junctions

Cell-to-cell attachment complexes can be subdivided into at least six categories based on their molecular components.¹⁰¹ In terms of the biology of stratified epithelium, the desmosome (macula adherentes) and the adherens junction are the key cell-to-cell adhesions. Desmosomes and adherens junctions have distinct cytoplasmic plaque components and adhesion glycoproteins.¹⁰²⁻¹⁰⁴ (The molecular constituents of these structures fall into two broad categories: (1) cadherin glycoproteins that are transmembrane molecules with both extracellular and intracytoplasmic domains (Fig 4-29), and (2) cytoplasmic proteins that make up desmosomal plaques associated with keratin filaments (Fig 4-30) and adherens junctional plaques with actin filaments (Fig 4-31). The desmosome-intermediate filament scaffold serves as a mechanical stabilizer of epithelial tissues, while the adherens junction-actin filament complex provides a system for generating tension across epithelial tissue. Defects in the desmosome-intermediate filament sys-

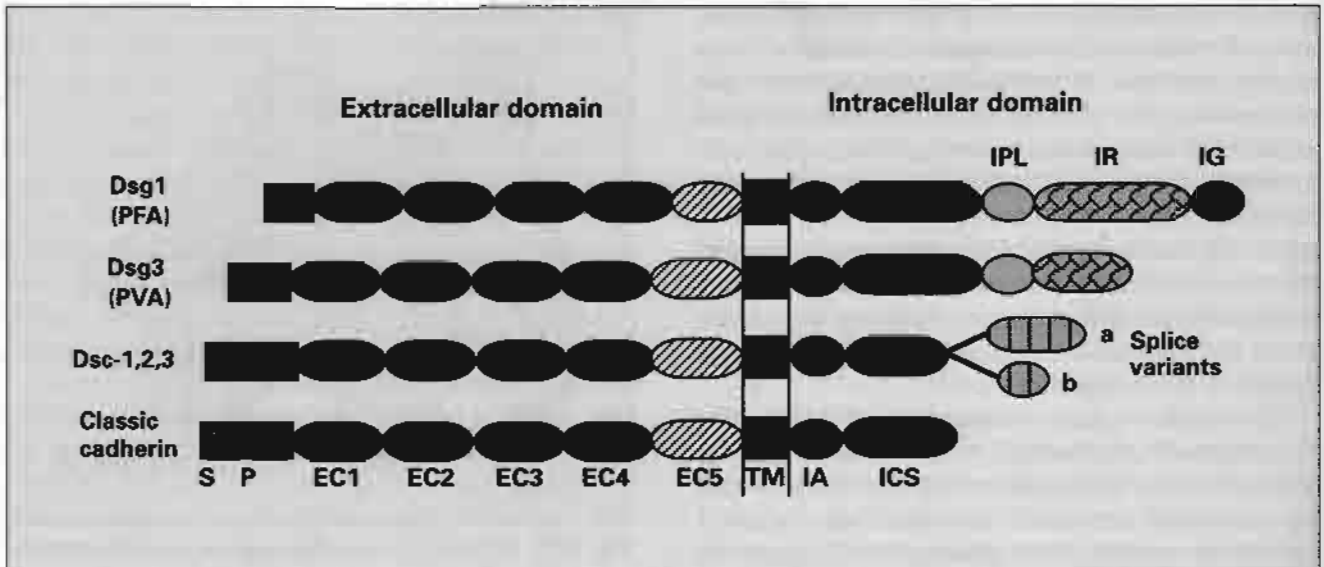


Fig 4-29 Molecular structure of cadherins. The family of epithelial cadherins includes classic cadherins found in adherens junctions and the desmosomal cadherins ([Dsc] desmocollins; [Dsg] desmogleins; [PFA] pemphigus foliaceus antigen; [PVA] pemphigus vulgaris antigen). Diversity resides mainly in the intracellular terminal domains. (S) Signal peptide; (P) prosequence; (EC) repeating extracellular domains; (TM) transmembrane segment; (IA) intracellular anchor; (ICS) intercellular space; (IPL) intracellular proline-rich linker; (IR) intracellular repeating domains; (IG) intracellular glycine-serine-rich domains. (Adapted from Amagai,¹⁰⁴ with permission from Blackwell Science.)

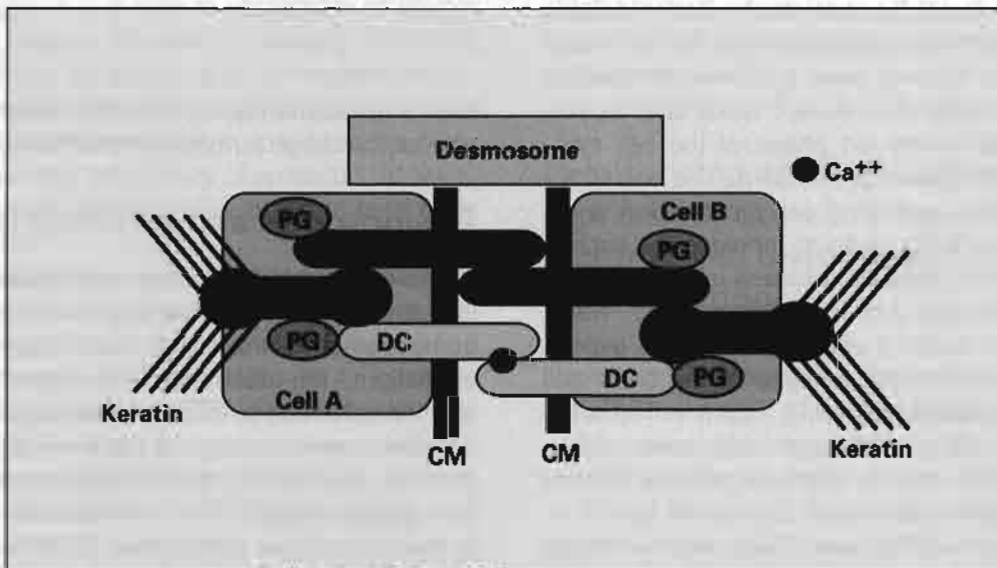


Fig 4-30 Cell-to-cell adhesion via the desmosome. Cell-to-cell desmosomal attachments of keratin filaments are effected via desmoplakin (DP) and the transmembrane proteins desmoglein (DG) and desmocollin (DC). Plakoglobin (PG) and DP are localized in the attachment plaque. The extracellular domains of DG and DC are bridged by calcium ions (red circles). (CM) Cell membrane. (Adapted from Amagai,¹⁰⁴ with permission from Blackwell Science.)

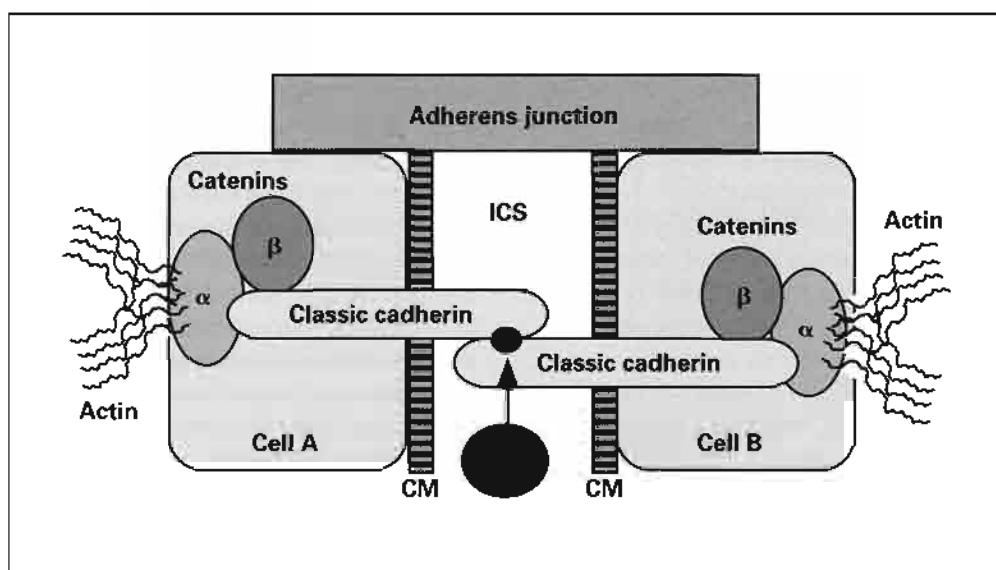


Fig 4-31 Cell-to-cell adhesion via the adherens junction. The adherens junction is an intercellular attachment site for actin filaments. It is formed by transmembrane cadherins of the classic (E type) and intracellular catenin proteins. The catenins form a thin, plaque-like aggregate along the inner leaflet of the cell membrane (CM). (ICS) Intercellular space. (Adapted from Amagai,¹⁰⁴ with permission from Blackwell Science.)

tem cause a variety of epidermal and mucosal blistering diseases (see "Disorders of epithelial attachment").

Of special significance to epithelial cell biology is the growing evidence that components of desmosomes and adherens junctions also act as sites of signal transduction.¹⁰³ Complex interactions between cell-to-cell adhesions and signal transduction pathways may provide key mechanisms for the control of tissue morphogenesis and cell migrations.¹⁰⁵

Desmogleins and desmocollins are transmembrane molecules of the cadherin family of adhesion proteins that contact the plaque material inside the cell and extend into the "cement" region of the intercellular space (see Figs 4-29 and 4-30).^{102,104} In the presence of extracellular calcium, the cadherins take part in homophilic binding across narrow intercellular spaces. Recent studies suggest that in the presence of calcium, desmocollins and desmogleins associate in heterophilic pairs via their outermost ectodomains in the extracellular space (in contrast to the homophilic pairs shown in Fig 4-30).¹⁰⁶ Desmogleins 1 and 3 are found almost exclusively in desmosomes of SSE and in tumors that arise from SSE.¹⁰² A third desmoglein isoform (type 2) is present in all tissues that form desmosomes.¹⁰⁷ Desmogleins 2 and 3 are expressed in basal and spinous cells, while desmoglein 1 appears in the stratum granulosum.¹⁰⁸

Desmoplakin, plakophilin 1, and plakoglobin are cytoplasmic proteins localized to the desmosomal plaque (see Fig 4-30). Desmogleins and desmocollins are linked to intermediate filaments (keratin) by the linker protein, desmoplakin. The carboxy terminal of desmoplakin is the binding site for keratin filaments.^{52,109} Plakophilin 1 also binds keratin filaments. It is added to desmosomal plaques as they mature, thereby increasing the stability of older desmosomes.¹¹⁰ Plakoglobin (also known as γ -catenin) is another member of the desmosomal plaque. It may have regulatory functions as well as serving as a link between desmoplakin and cadherin components.¹¹¹ Several accessory plaque proteins are believed to account for the cell-type specificity and signaling properties of desmosomes.¹⁰¹

Phosphorylation-dephosphorylation reactions regulate desmosomal plaque assembly from soluble precursors. Soluble plakoglobin is phosphorylated on serine and threonine. Dephosphorylation favors association of plakoglobin with desmoglein 1 and E-cadherin to form insoluble attachment plaques¹¹² and the association of keratin to desmoplakin.¹⁰⁹ Inhibition of protein phosphatases leads to desmosome disassembly and reorganization of keratin filament networks.¹¹³ Phosphorylation of the carboxy terminal of desmoplakin by protein kinase A destabilizes the linkage of keratin to desmoplakin.¹⁰⁹ The

phosphorylation and dephosphorylation events carried out by kinases and phosphatases, respectively, are interrelated and regulated by signaling cascades responsive to levels of extracellular calcium.¹⁰⁹ For example, the inhibition of protein kinase C leads to increased numbers of desmosomes.¹¹⁴

Epidermis and human oral mucosa also contain focal cell-to-cell and cell-to-matrix (basal lamina) adherens junctions. They have been identified by immunofluorescence localization of α -catenin, plakoglobin, and associated actin filaments. In adherens cell-to-cell junctions, the linkage between the actin filaments of the cytoskeleton and classic E- and P-cadherins is made by α -catenin, a protein with homology to vinculin of focal adhesions (see Fig 4-31). Plakoglobin binds α -catenin in the adherens attachment plaque.^{101,115,116} Basal cells express both E- and P-cadherins, while upper-level keratinocytes express only E-cadherins.¹¹⁷ E-cadherin is essential for growth regulation and stratification of gingival and epidermal cells.^{118,119} The assembly of adherens junction plaque proteins is also favored by phosphatase activity. Phosphorylation of tyrosine on E-cadherins and β -catenin causes disassembly of adherens junctions.¹²⁰

In the electron microscope, adherens junctions are distinguished from desmosomes by the absence of a central dense line in the intercellular space and by thinner, less-defined attachment plaques.¹²¹ The co-participating cell membranes forming adherens junctions are evenly spaced at roughly the same distance as in desmosomes, and the adjacent cytoplasm contains fine actin filaments and a rather thin layer of granular attachment material juxtaposed to the inner leaflet of the cell membrane. Transmembrane proteins of the classic E-cadherin type bridge the intercellular space and, by association with α -catenins, bind cytoplasmic actin filaments (see Fig 4-31).¹⁰⁴

Antibodies to classic cadherins of adherens junctions block desmosome formation and cell stratification, indicating that adherens junction formation may be the first step in epithelial cell-to-cell adhesion. Experiments suggest that the formation of desmosomes is dependent on prior formation of adherens junctions.¹²² The stimuli for desmosome assembly are generated by signaling cascades triggered by adherens junction-plakoglobin binding reactions.

In addition to cadherins, calcium is required for the formation of desmosomes and adherens junctions.¹²³ Sequestration of calcium by exogenous chelating agents causes newly formed epithelial cell aggregates to disassociate and their desmosomes to undergo internalization and digestion.¹²⁴ Subsequent

exposure of the dispersed epithelial cells to a normal calcium concentration leads to the reappearance of adherens junctions and desmosomes.

In disassociated cells in low-calcium media, desmogleins are evenly dispersed on the cell membrane. When calcium is added to the media, desmogleins aggregate rapidly to form desmosomes, and there is a concomitant rearrangement of keratin intermediate filaments from a perinuclear distribution to a desmosomal association.¹²³ New desmosomes are formed from soluble precursors without the need for protein synthesis. However if more than 8 hours elapses between the removal of calcium and its reintroduction, synthesis of new desmosomal subunits is required. Thus, the soluble precursor pool of desmosomal proteins is turned over *in vitro* within 8 hours.

When epithelial cells are stabilized into mature cell sheets, they resist disassociation induced by low-extracellular calcium concentration.¹⁰³ This condition is thought to be the result of additional adhesive interactions provided by desmosomal noncadherin-type proteins.

Under the influence of certain cytokines and growth factors released during inflammation and wound healing, desmosomes revert back to the calcium-dependent state. In the calcium-dependent state, desmosomes are more rapidly disassembled and assembled, creating a condition favorable to cell migration.¹⁰³ Hepatocyte growth factor, a product of fibroblasts, causes desmosome disassembly by stimulating the activity of protein tyrosine kinase.¹²⁵

Basal attachment apparatus

Basal cells of stratified squamous epithelia are bound to underlying connective tissue by an attachment apparatus comprising cytoplasm, plasma membrane, and extracellular proteins (Fig 4-32). Nondividing basal cells of SSE often develop major cell processes (pedicles) that project into the connective tissue, thereby increasing the surface area for attachment (see Fig 4-4). Pedicles are plentiful in areas of the skin and mucous membranes (such as the oral gingival epithelium and hard palate) that are exposed to high shearing forces.

Molecular associations for attachment are concentrated in buttonlike structures, the hemidesmosomes. They serve as sites for binding keratin intermediate filaments to the basal lamina via several plaque and transmembrane proteins. The basal lamina is in turn joined to collagen fibers by a system of anchoring fibrils (type VII collagen) and connective tissue anchoring plaques (see Fig 4-32).

The basal lamina densa, a dense network of collagen type IV, laminin, nidogen (entactin), and heparan sulfate proteoglycan (perlecan), forms adjacent to the basal surface of the basal cells (Fig 4-33).^{118,126-128} It was previously thought that the basal lamina consisted of two components: the lamina lucidum and the lamina densa. The presence of the lamina lucidum, a clear zone between the lamina densa and the cell membrane, was problematic, because it raised doubts about potential molecular interaction between cell membrane constituents and the extracellular matrix components that make up the lamina densa. It is now known that the lamina lucidum is an artifact formed during the dehydration phase of routine tissue preparation.¹²⁹ In specimens prepared by freeze-fixation and freeze-substitution, the lamina densa is in direct juxtaposition to the cell membrane. This finding demonstrates that the extracellular domains of the integrin molecules are sufficiently close to the lamina densa to permit matrix-receptor interactions.

The lamina densa is constructed of type IV collagen molecules assembled to form a meshwork whose pore size ranges from 8 to 20 nm, depending on the epithelial site (see Fig 4-33).¹²⁷ Laminin 1 also undergoes self-assembly to form a meshwork. The laminin mesh forms without covalent bonding and thus is less stable than the collagen IV network. The two networks combine through the interaction of nidogen bridges to create a scaffold containing other constituents, such as perlecan, fibronectin, and other glycoproteins.^{127,130}

When visualized in specimens prepared by quick-freezing and deep etching, the lamina densa consists of numerous closely packed granules in a three-dimensional network of type IV collagen filaments. These granules consist of laminin, nidogen, fibronectin, and perlecan.¹³¹ Fluorescence immunocytochemistry has proven especially useful for localizing these molecules to the lamina densa.

The biochemical bond formed between integrins and laminin 5 in the basal lamina densa provides a firm attachment of the basal cells to the extracellular matrix. Integrin receptors for collagen and laminin 1 ($\alpha 2\beta 1$ and $\alpha 3\beta 1$) and for laminin 5 ($\alpha 3\beta 1$, $\alpha 6\beta 1$, and $\alpha 6\beta 4$) are inserted into the basal plasma membrane.^{127,132,133} The $\alpha 5\beta 1$ receptor for fibronectin and the $\alpha v\beta 5$ receptor for vitronectin are also expressed in basal cells. Interactions between $\alpha 3\beta 1$ and laminin 5, and between $\alpha 5\beta 1$ and fibronectin, lead to the formation of focal adhesions. These are points of anchorage between the extracellular matrix and the actin microfilament network.

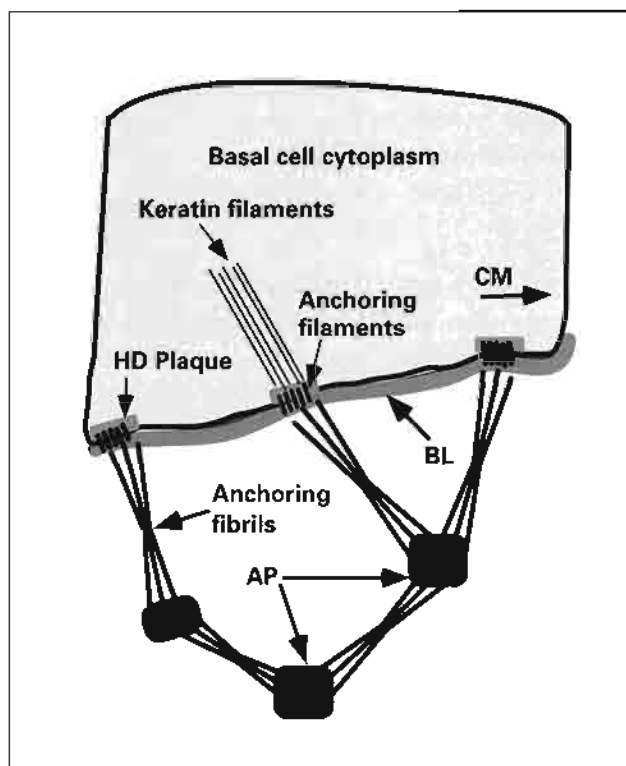


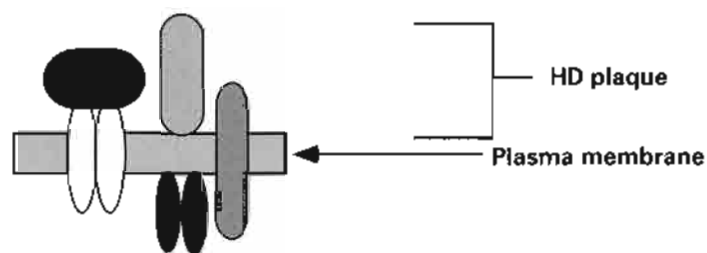
Fig 4-32 Anchoring apparatus beneath basal cells. Keratin filaments insert into desmosomal plaques. Anchoring filaments made up of laminin 5 (kalinin) form bridges between hemidesmosomal (HD) plaques and the basal lamina (BL), where they terminate adjacent to the insertion of anchoring fibrils. Anchoring fibrils also bind to anchoring plaques (AP) in the connective tissue, forming looplike structures that intertwine with type I and type III collagen fibrils. (CM) Cell membrane.

Deficiency of $\alpha 3\beta 1$ expression leads to disorganization of the basement membrane and rupture of the epidermal-dermal connection.⁹⁰

Intercellular adhesion of basal layer keratinocytes along their lateral cell surfaces is mediated by cadherins and by direct binding of $\alpha 2\beta 1$ to $\alpha 3\beta 1$ integrins. Association of $\alpha 6\beta 4$ with laminin 5 in hemidesmosomes anchors keratin intermediate filaments to the underlying connective tissue via collagen type VII. The bridge between cell and matrix is completed when the amino terminal domain of laminin 5 binds to type VII collagen.¹³⁴

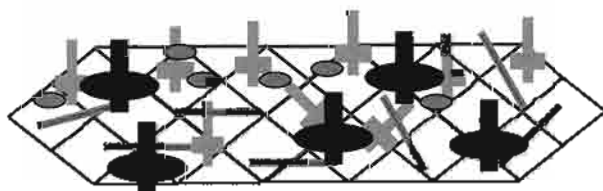
In addition to acting as molecular bridges between cytoskeletal proteins and the extracellular matrix, the integrins also activate signaling pathways via tyrosine phosphorylation cascades.¹³⁵ It appears that integrin-laminin 1 signaling interactions at the basal cell surface regulate cell polarization and cell differentiation programs in epithelial cells.

Hemidesmosome



-  **BPAG-1:** Bullous pemphigoid antigen 1. High-sequence homology to desmoplakin. It mediates the attachment of keratin filaments to the hemidesmosomal plaque.
-  **Plectin:** Plaque matrix protein with homology to desmoplakin and BPAG-1.
-  **BPAG-2:** Bullous pemphigoid antigen 2. Extracellular collagenous sequence (collagen type XVII). Attachment to other proteins of the basal lamina. May form part of the anchoring filaments.
-  **$\alpha 6 \beta 4$ Integrin:** Attachment to laminin 5 (kalinin, epiligrin). Contributes to formation of anchoring filaments.
-  **Uncein and ladinin:** Newly identified components of the anchoring filaments.

Basal lamina complex



-  **Perlecan:** Heparan sulfate proteoglycan.
-  **Nidogen (entactin):** Matrix adhesion molecule. Binds laminin to collagen type IV.
-  **Collagen type IV:** Assembles into a macromolecular fabric, forming the meshwork of the basal lamina densa.
-  **Laminin 1 and laminin 5 (kalinin, epiligrin):** Attaches to integrin $\alpha 6 \beta 4$. Contributes to formation of anchoring filaments.
-  **Fibronectin:** Matrix adhesion molecule. Binds to collagen, nidogen, and integrins ($\alpha 2 \beta 1$, $\alpha 3 \beta 1$).

Fig 4-33 Molecular components of the hemidesmosomal (HD) attachment plaque and the underlying basal lamina densa.

Laminin 5 appears to play a key role in initiating hemidesmosome assembly.¹³⁶ When laminin 5 is added to carcinoma cells, they form ordered arrays, and cultured epithelial cells adhere to the substratum. Thus, $\alpha 6 \beta 4$ mediates anchorage and $\alpha 3 \beta 1$ mediates motility on laminin 5. The latter interaction assumes greater significance during wound healing, when epithelial cells must migrate to cover the wound bed.

Keratin filaments are bound to the hemidesmosome by plectin and BPAG-1.¹²⁸ An additional protein, intermediate filament attachment protein 300, has been localized to the cytoplasmic edge of the attachment plaque. Another transmembrane attachment component of the hemidesmosome is the BPAG-2 transmembrane protein (see Fig 4-33).¹²⁸ This protein contains collagenous sequences and is now classified as type XVII collagen. Anchoring filaments formed by laminin 5, collagenous domains of BPAG-2, uncein, and laminin course from the hemidesmosomes to the basal lamina (see Fig 4-32). These filaments are clearly visible in routinely prepared specimens as they cross the lamina lucida.

In addition to their adhesion function, the integrins have a role in keratinocyte proliferation and differentiation. The observation of an inverse relationship between integrin ($\alpha 6 \beta 4$) expression and keratinocyte terminal differentiation suggests that integrin-ligand-mediated signaling mechanisms might retard the onset of keratinocyte differentiation. During terminal differentiation of keratinocytes, the expression of $\beta 1$ integrins is also reduced. Epidermal stem cells have been shown to express high levels of $\beta 1$. It is noteworthy that keratinocytes in suspension, which normally undergo terminal differentiation, can be blocked from doing so by the addition of integrin ligands such as collagen type IV to the culture fluid.

Oral squamous carcinoma cells demonstrate reduced expression of $\alpha 2$, $\alpha 3$, $\alpha 6$, and $\beta 4$ integrin subunits, thereby decreasing their adhesion to basal laminae and enhancing their ability to invade connective tissues. A direct correlation between epithelial dysplasia and the loss of basal lamina components has been reported in oral squamous carcinomas.¹³⁷

Type VII collagen forms special anchoring fibrils that bind to laminin 5, to collagen type IV, and to anchoring plaques in the lamina propria.¹³⁸ Looplike formations of type VII collagen fibrils originate and terminate in the lamina densa, thereby providing additional anchorage by interdigitating with types I and III collagen fibrils (see Fig 4-32).

Fibronectin filaments deposited in subepithelial connective tissue participate in adhesive associations with type VII collagen fibrils and the basal lamina.¹³⁸ Immunohistochemical studies of fibronectin in oral mucosa indicate that it is present in its highest concentration in palate and tongue connective tissue, often appearing to have a netlike distribution.¹³⁹

Collagen types VI and XV are also localized to the basement membrane zone, where they appear to provide additional adherence between the epithelium of skin and oral mucosa and the underlying connective tissue.^{140,141} Fibrillin, a matrix protein that forms microfibrils about 10 to 12 nm wide, also participates in the attachment of epidermis to connective tissue. The fibrillin microfibrils form a network that projects downward from the collagen VII anchoring fibrils.¹⁴²

Type IV collagen

Type IV collagen is a nonfibrillar collagen that constitutes the major fraction of the basal lamina densa. Type IV procollagen molecules are heterotrimers assembled from six genetically distinct α (IV) chains. Each chain consists of a long linear collagenous domain of about 1,400 amino acids. Several short, non-collagenous segments that impart flexibility to the molecule interrupt the collagenous domain. The most common heterotrimer is made up from two $\alpha 1$ (IV) chains and one $\alpha 2$ (IV) chain.

The triple helical type IV procollagen molecules undergo self-assembly to form the basal lamina.¹⁴³ Networks are assembled by the formation of collagen type IV dimers and type IV tetramers by amino terminal interactions.¹⁴⁴ Network building continues as the helical domains of adjacent type IV collagen molecules align laterally to form suprahelices and to bring globular carboxy terminals into close contact. The aggregation of contiguous globular carboxy domains is stabilized by the formation of disulfide bridges.¹⁴⁴ The resulting supramolecular aggregate resembles a porous, fishnetlike material that serves as a scaffold for intermolecular association with other components of the basal lamina (see Fig 4-33).

The collagenous segments of the type IV procollagen molecules are extensively glycosylated. The many disaccharide side chains are important in intermolecular interactions with other constituents of the basal lamina, such as heparan sulfate proteoglycan, nidogen, fibronectin, and BPAG-2.

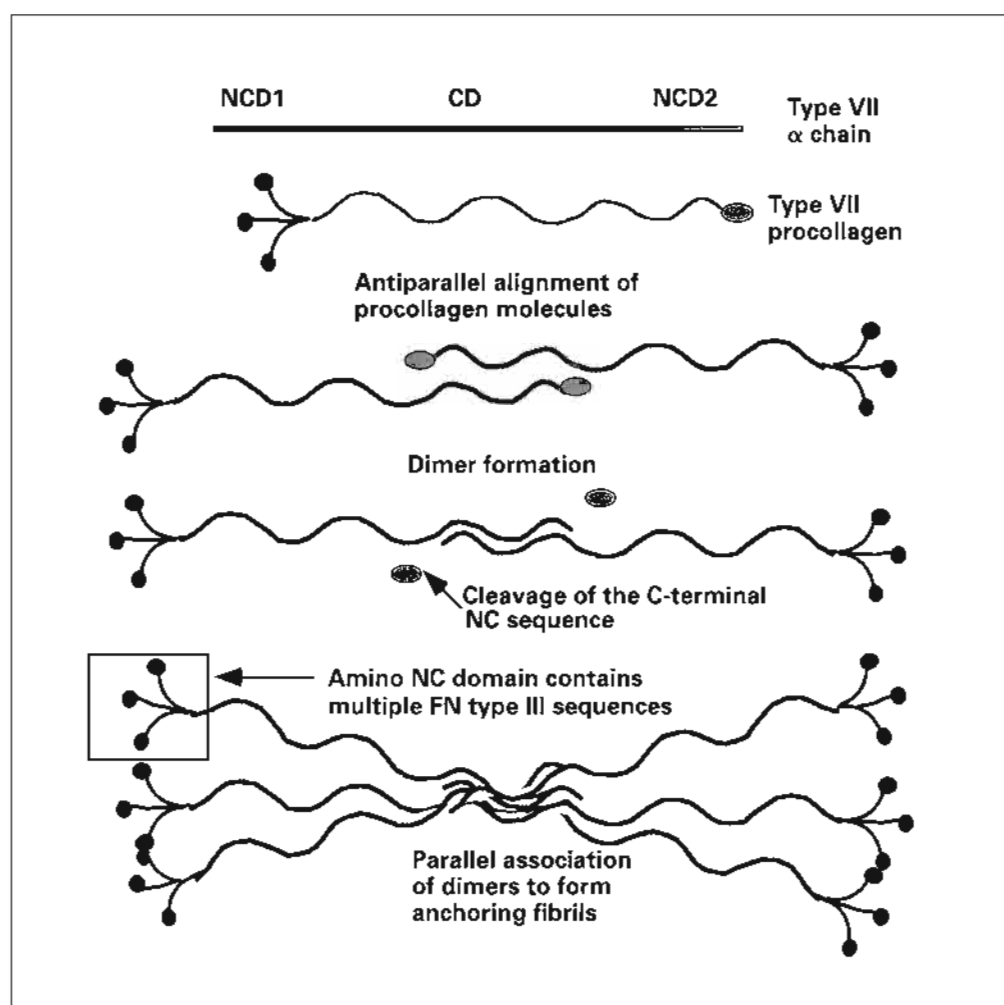


Fig 4-34 Steps in the assembly of anchoring fibrils through the association of type VII collagen molecules. (NCD) Noncollagenous domain; (CD) collagenous domain; (C-terminal) carboxy terminal; (NC) noncollagenous; (FN) fibronectin.

Type VII collagen

Type VII procollagen molecules are composed of three identical α chains. Each chain contains a central collagenous domain and two noncollagenous terminal domains. The collagenous segments of three α chains associate in a triple helical arrangement to form a type VII procollagen molecule (Fig 4-34). Following cleavage of the carboxy noncollagenous terminal domains, dimer formation occurs by side-to-side aggregation of partially overlapping procollagen molecules.

The lateral assembly of collagen type VII dimers forms anchoring fibrils. The anchoring fibrils have protruding amino terminal domains (noncollagenous domain 1) that contain multiple fibronectin type III sequences.¹⁴⁵ Noncollagenous domain 1 allows type VII collagen to bind to type IV collagen in the basal

lamina and in the anchoring plaques of the basement membrane connective tissue. Collagen type VII also binds to fibronectin by interaction with its collagen-binding domain.¹³⁸ Looplike formations of type VII collagen fibrils originate and terminate in anchoring plaques and the lamina densa, thereby providing additional anchorage by interdigitating with types I and III collagen fibrils (see Fig 4-32).

Type VII collagen is made by epithelial cells and by fibroblasts of the lamina propria. Monocultures of both cell types produce small quantities of type VII collagen; however, cocultures of epithelial cells and fibroblasts produce larger amounts of the protein, indicating that epithelial-mesenchymal interaction is necessary for the efficient production of anchoring fibrils.¹⁴⁶ The expression of type VII collagen is strongly dependent on the stimulatory effect of TGF- β .

Abnormal type VII collagen leads to junctional blistering diseases (see "Disorders of epithelial attachment"). Increased expression of type VII collagen, with formation of unusually high numbers of anchoring fibrils, occurs in systemic sclerosis.¹⁴⁷

Cell surface proteoglycans (syndecan and epican)

Oral keratinocytes synthesize extracellular and cell-associated heparan sulfate-rich proteoglycans.¹⁴⁸ Syndecans are a family of cell surface proteoglycans expressed in all cell types (Fig 4-35). Four genetic types, with different tissue distribution patterns, have been identified: syndecan 1 (the original syndecan), syndecan 2 (fibroglycan), syndecan 3 (*N*-syndecan), and syndecan 4 (amphiglycan).^{149,150} Syndecan 1 is strongly expressed in the stratum spinosum of stratified squamous epithelia but present in only small amounts in the basal cell layer.¹⁵¹ The syndecan molecule is anchored to the cell membrane by a transmembrane hydrophobic amino acid sequence. The short cytoplasmic domain (about 28 to 34 amino acids) contains four tyrosine residues, targets for tyrosine kinases and potentially important in starting signaling cascades.¹⁴⁹

The extracellular domain is the largest part of the molecule and the site of diversity. Serine-glycine pairs on the polypeptide backbone provide points for the attachment of glycosaminoglycan side chains. Chondroitin sulfate and heparan sulfate chains are attached to syndecan 1. Biochemical studies have shown that different cell types produce syndecans with different glycosaminoglycan structures.¹⁵² Glycosaminoglycan structural diversity leads to differences in binding affinities to matrix molecules and growth factors. Via its glycosaminoglycan side chains, syndecan 1 is capable of forming attachments to fibronectin; collagen types I, III, and V; and tenascin.¹⁴⁹ The heparan sulfate side chains also serve as binding sites for growth factors.

Epithelial cells lose their epithelioid configuration and assume a fusiform shape when syndecan expression is abolished.¹⁵³ On restoration of syndecan expression, the cells regain their epithelial shape. Changes in cell shape in response to syndecan expression are believed to be due to the effect of syndecan on the binding affinity of receptors for extracellular adhesion proteins. Syndecan 4, along with actin, integrins, and fibronectin, localizes to developing focal adhesions.¹⁴⁹ Many of the extracellular ma-

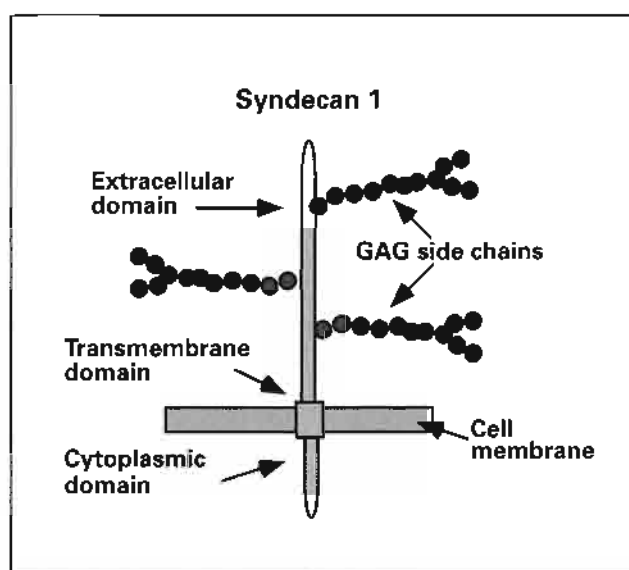


Fig 4-35 Domains of the syndecan 1 molecule. (GAG) Glycosaminoglycan.

trix proteins that bind to integrin receptors also bind to the heparan sulfate subunits of the syndecans.

Increasing evidence points to a role for syndecan as a coreceptor in signal transduction events. Syndecan potentiates signaling by binding fibroblast growth factor 2, HB-EGF, and vascular endothelial cell growth factor. Growth factor potentiation requires that the syndecan remain intact as an integral membrane protein and that it be in juxtaposition to the receptor protein (Fig 4-36). Syndecan 1 expression is decreased on keratinocytes of stratified squamous cell carcinomas (SCCs). Those SCCs that show some staining for syndecan 1 appear to have a better prognosis.¹⁵⁴

The extracellular domain of syndecan is susceptible to proteolytic cleavage by extracellular matrix metalloproteinases. Cleavage of the molecule occurs close to the cell membrane that is releasing the proteoglycan moiety into the extracellular space. Soluble glycosaminoglycans (especially heparan sulfate) that have been shed from the cell surface can bind and sequester growth factors, thereby decreasing the concentration of growth factor available for interaction with its receptor. Thus, variation in syndecan expression and shedding may have regulatory effects on cellular responses to growth factors. Most of the evidence for this concept comes from studies of basic fibroblast growth factor, basic fibroblast growth factor receptor, and their interaction with syndecan heparan sulfate.

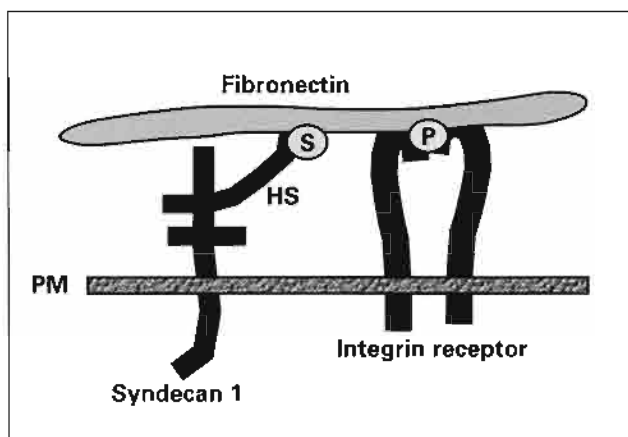


Fig 4-36 Association of syndecan and integrin in fibronectin binding. The integrin receptor engages the primary (P) site, while a contiguous syndecan molecule engages a secondary (S) binding site via heparan sulfate (HS). The binding of syndecan adds to the strength of the attachment and presumably to the resulting level of signaling. (PM) Plasma membrane.

Epican is similar to syndecan, in that it carries heparan sulfate and chondroitin sulfate glycosaminoglycan side chains on its extracellular domain. It is a form of CD44 cell surface protein, expressed primarily by epidermal cells. It plays a role in mediating keratinocyte cell-to-cell adhesion in a low-avidity, hyaluronan-dependent, calcium-independent attachment mechanism. Integrins and cadherins form the molecular basis of the high-avidity adhesion system between keratinocytes. The syndecan and epican proteoglycans belong to the mucopolysaccharide intercellular "cement" described in the older scientific literature.

Noncollagenous components of the basal lamina

Nidogen (entactin)

The nidogen molecule is rod shaped with globular domains at both the carboxy and amino terminals. Nidogen binds to type IV collagen, laminin, and perlecan.^{127,130,155} Because laminin does not have the ability to bind to collagen type IV, nidogen appears to play a crucial role in the assembly of the basal lamina by acting as a bridge between collagen type IV and laminin. At the epithelial-mesenchymal junction, nidogen is localized in the basal lamina and attachment plaques. In vitro studies indicate that, although fibroblasts produce nidogen, the assembly of a basal lamina requires participation of epithelial cells.¹⁵⁶

Perlecan

Perlecan is a large proteoglycan (400 to 450 kDa) found within basal laminae, including those of the oral mucosa.¹⁵⁷ The core protein consists of five domains (Fig 4-37).¹⁵⁷ Domain 1, at the amino terminal, is unique to perlecan and is a site for attachment of heparan sulfate side chains. Domain 2 shares homology with the low-density lipoprotein receptor. Domain 3 shares homology with the short arm of the γ chain of laminin. Domain 4 contains 21 immunoglobulin-like repeats similar to the neural cell adhesion molecule. Domain 5 contains EGF-like repeats and shares homology to the laminin α chain.

The main producers of perlecan are fibroblasts adjacent to the basal lamina. Perlecan binds to other molecules of the basal lamina such as fibronectin, nidogen, and laminin. The orientation of the perlecan molecule in the basal lamina, deduced from immunocytochemical studies, indicates that the amino terminal (domain 1) is positioned near the plasma membrane of the basal cell.

In addition to its structural contribution to the basal lamina, perlecan has significant interactions with growth factors. Growth factors such as basic fibroblast growth factor and TGF- β are bound and stored at the heparan sulfate chains of perlecan.^{158,159} Release of basic fibroblast growth factor via the action of heparanase occurs during wound healing.

Laminins

Laminins are large, extracellular, cross-shaped adhesion proteins consisting of three polypeptide chains (α , β , and γ).^{155,160} The α chain is the largest of the three. It has three globular domains separated by numerous EGF repeats in its amino terminal (Fig 4-38). A large component consisting of five subunits characterizes its rather large carboxy terminal. The smaller β and γ chains contain two domains in the amino terminal. The α -helical domains of the three chains form a coiled-coil structure stabilized by inter-chain disulfide bridges.

At least eight different genetic polypeptide chains and seven distinct heterotrimeric molecular assembly patterns have been characterized.^{127,155} Laminin 1 is a major component of most basal laminae. It is a ligand for $\alpha 3 \beta 1$ integrin receptor. The location of binding sites for perlecan, nidogen, various integrins, and other molecules to laminin are shown in Fig 4-38.

Laminin 1 plays an essential role in the assembly of basal laminae, as well as in the adhesion of cells to the basal lamina.¹⁶¹ Laminin 5 (kalinin) and laminin 6 form anchoring filaments that join hemidesmosomal attachment plaques to the basal lamina densa.

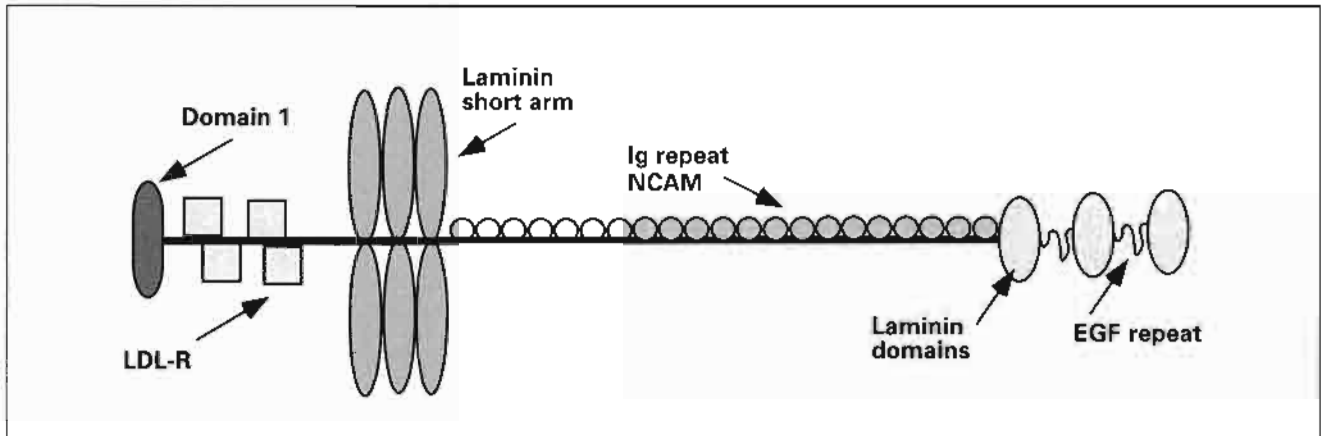


Fig 4-37 Structure of the extracellular proteoglycan, perlecan. The polypeptide has five domains with homologies to other proteins, as indicated. Heparan sulfate chains (not shown) are attached at domain 1. (LDL-R) Low-density lipoprotein receptor; (Ig) immunoglobulin; (NCAM) neural cell adhesion molecule; (EGF) epidermal growth factor. (Adapted from Iozzo et al,¹⁵⁷ with permission from Portland Press.)

The integrins $\alpha 3\beta 1$, $\alpha 6\beta 1$, and $\alpha 6\beta 4$ act as receptors for laminins 1 and 5.^{127,162} Laminin 5 accelerates anchorage of epithelial cells to extracellular matrix and the formation of hemidesmosomes.¹³⁶ During wound healing, laminin 5 promotes cell migration by its interaction with the $\alpha 3\beta 1$ integrin receptor.¹⁶³ Stabilization of epithelial cells occurs when laminin 5 and the $\alpha 6\beta 4$ integrin interact to form part of the hemidesmosomal complex.

Small peptide fragments of laminin have been shown to have significant biologic activities. They have been shown to promote tumor growth, angiogenesis, and nerve regeneration. The hope is that, in the future, clinical use of such peptides might accelerate wound healing.

Epithelial-mesenchymal interactions

Oral epithelium demonstrates regionally specific patterns of cytodifferentiation and morphogenesis, from simple noncornified epithelium of the ventral surface of the tongue to more complex patterns that determine the structure of the filiform appendages of the dorsal surface of the tongue. These patterns are preserved over the life span of the organism, despite continued and rapid turnover of the cell populations. Whether or not these epithelial site-specific differences are the sole result of epithelial genotypic expression or the result of continued epithelial-mesenchymal interactions is a question that has intrigued experimental biologists for many years. There are numerous examples of necessary epithelial-mesenchymal interactions in embryonic develop-

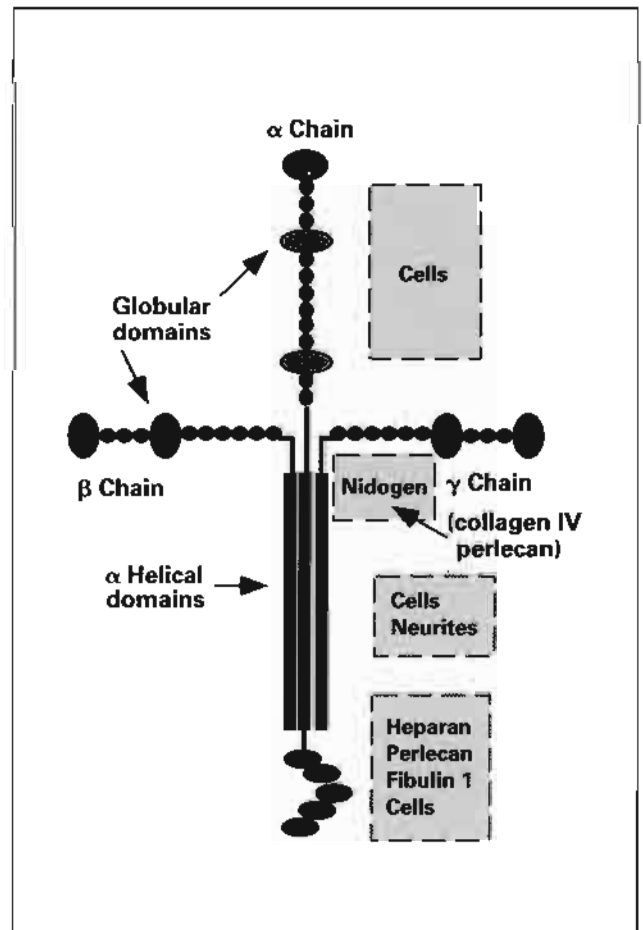


Fig 4-38 Structure of the laminin molecule, which consists of three polypeptide chains. Binding regions for cells (arginine-glycine-aspartic acid sequences) and for various extracellular matrix molecules are shown. The interaction between laminin and type IV collagen is mediated via the nidogen. (Adapted from Timpl and Brown,¹²⁷ with permission from Elsevier Science.)

Figs 4-39a to 4-39d Oral epithelial-mesenchymal tissue recombinations cultured for approximately 3 weeks. (Reprinted from Mackenzie and Hill,¹⁶⁵ with permission from Springer-Verlag. Original magnification $\times 300$.)

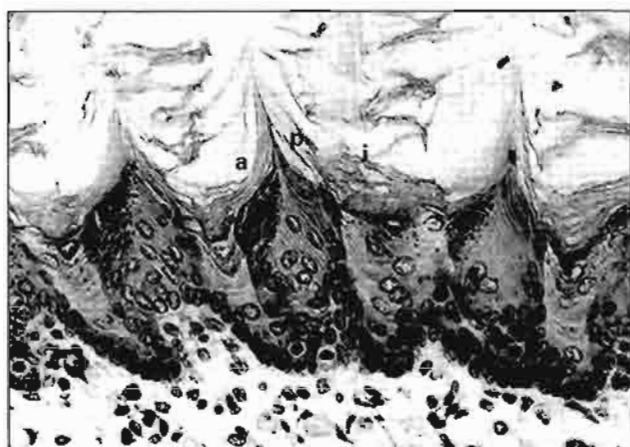


Fig 4-39a Self-recombination of tongue epithelium and connective tissue, illustrating the maintenance of tongue papillary architecture and the characteristic anterior (a), posterior (p), and interpapillary (i) patterns of cornification.

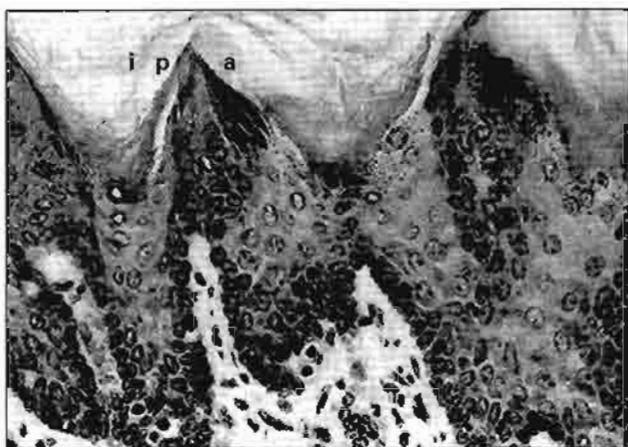


Fig 4-39b Palate epithelium recombined with tongue connective tissue. The epithelium adopted a papillary configuration with acquisition of anterior (a), posterior (p), and interpapillary (i) cornification patterns.

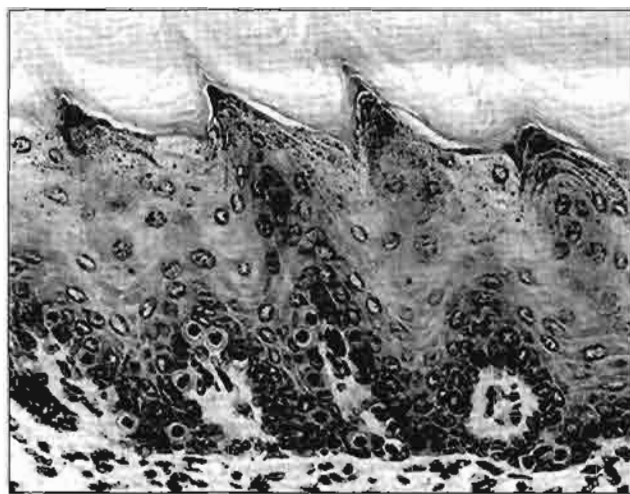


Fig 4-39c Buccal epithelium combined with tongue connective tissue. Note the acquisition of a papillary architecture but with less pronounced "spike" formation and with less distinct anterior and posterior cornification patterns.

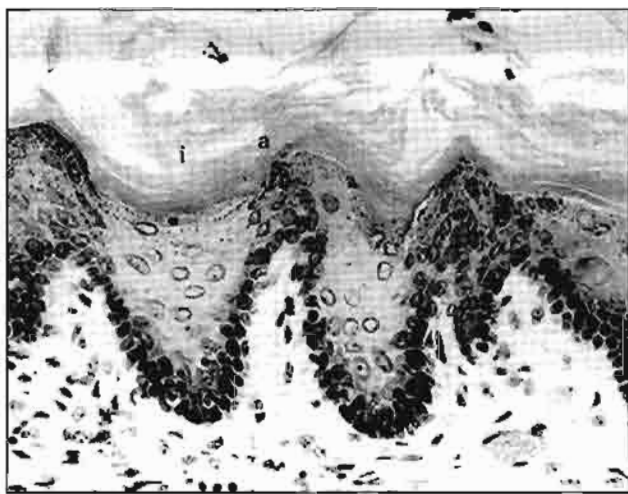


Fig 4-39d Tongue epithelium grown on palatal connective tissue. There was a degree of retention of the interpapillary (i) and anterior (a) patterns of cornification, but the regularity of the papillary architecture was much reduced from normal.

ment—tooth formation constitutes a prime example—but their importance in adult tissues, such as oral mucosa, is less clear.

The development of techniques for separating epithelium from its mesenchyme and for their heterotypic recombination and subsequent culture gave experimental biologists the tools to begin elucidating epithelial-mesenchymal interactions.¹⁶⁴ The first lesson learned from these studies was that survival of epithelial tissues requires a supportive matrix as well as soluble substances of connective tissue cell ori-

gin. These are often provided in culture systems by including a fibroblast "feeder layer" on which the epithelial cells are grown. In epithelial-mesenchymal recombinations, these factors are provided by the connective tissue component.

In general, the epithelial tissues maintain the same pattern of cytodifferentiation characteristic of their site of origin when grown in combination with heterogeneous connective tissue.^{165,166} In such cases, the connective tissue is said to have a *permissive effect* on the epithelium. However, there are combina-

tions in which the differentiation pattern of the epithelium changes to reflect the site of origin of the connective tissue.¹⁶⁷ In such cases, the mesenchyme is said to have an *instructive influence* over the epithelium. For example, when fully cornified epithelium of the hard palate is grown in contact with the connective tissue lamina propria from the dorsum of the tongue, the palatal epithelium gradually assumes a heterogeneous differentiation pattern characteristic of filiform papillae (Figs 4-39a to 4-39d).¹⁶⁵ In this example, the lingual mesenchyme exerts an instructive influence over the palatal epithelium.

It has become increasingly clear that cytokines and growth factors arising in the supporting connective tissue control, in part, variations in gene expression that are responsible for epithelial tissue heterogeneity. In fact, there is a constant interplay, or cross talk, mediated by soluble factors emanating from and acting on both tissues. This interplay of epithelial and mesenchymal signals controls embryonic development as well as the maintenance of the adult phenotype.

Clinical Correlations

Disorders of epithelial attachment

A variety of diseases result from abnormalities in the molecules that constitute the epithelial attachment (Fig 4-40).^{168,169} Point mutations in the amino acid sequence of the rod domain of K5, K14, and K10 are the cause of two forms of blistering disease.¹⁷⁰ These conditions are usually inherited as autosomal-dominant traits.

In epidermolysis bullosa simplex, abnormal K5 and/or K14 chains weaken filament bundles anchored to desmosomes and hemidesmosomes.^{171,172} As a consequence, basal cells are less able to resist shearing forces and they undergo cytolysis with blistering of the epidermis and oral mucosa.

In epidermolytic hyperkeratosis, the mutation occurs in the K10 keratin molecule, thereby affecting the integrity of filament networks in suprabasal cells of cornifying SSE. Cytolysis of upper-level cells in the stratum spinosum leads to blistering of the epithelium as well as hyperproliferation of basal cells (probably by removal of feedback inhibition).

Genetic analyses of patients with epidermolysis bullosa simplex and those with epidermolytic hyperkeratosis indicate that the mutations involve a single amino acid. In most cases, the replacement of a single arginine residue by either cysteine or histidine is sufficient to alter the keratin network.

Genetic disorders

Dystrophic epidermolysis bullosa: Abnormal collagen type VII. Weak anchoring fibrils.

Junctional epidermolysis bullosa: Genetic mutations in laminin 5. Weak anchoring filaments. In another variant of this type, hemidesmosomes are either absent or rudimentary because of decreased expression of BPAG-2.

Epidermolysis bullosa simplex: Mutations in K5 and/or K14. Weakened filament bundles. Plectin abnormalities.

Epidermolytic hyperkeratosis: Mutation in K10 affecting filament network of suprabasal cells. Upper level blisters and hyperproliferation of basal cells.

Autoimmune disorders

Cicatricial pemphigoid: Autoantibodies to laminin 5.

Bullous pemphigoid: Autoantibodies to BPAG-1 and BPAG-2.

Pemphigus vulgaris: Autoantibodies to desmoglein 3.

Pemphigus foliaceus: Autoantibodies to desmoglein 1.

Fig 4-40 Diseases of epithelial attachment (blistering diseases). (BPAG-2) Bullous pemphigoid antigen 2; (K) keratin (K5, K10, K14).

In dystrophic epidermolytic bullosa, the inherited defect results in the production of abnormal type VII collagen.¹⁷³⁻¹⁷⁷ Blistering occurs as a result of a decreased integrity of the anchoring fibrils. Ultrastructural study of basal cells from blister sites reveals intracellular accumulation and degradation of type VII collagen and fewer extracellular anchoring fibrils.¹⁷⁸ Anchoring fibrils are also affected adversely in an autoimmune form of epidermolysis bullosa, in which immunoglobulin G antibodies are directed against the fibronectin type III sequences in the noncollagenous 1 domain of collagen type VII.

Junctional epidermolysis bullosa is a blistering disease caused by defects in laminin 5, BPAG-2, or $\beta 4$ integrin.^{175,177,179-181} Abnormalities in any one of these proteins can lead to faulty hemidesmosomal structure and a weakened epithelium-connective tis-

sue attachment. In patients with junctional epidermolysis bullosa, enamel dysplasia is also present, presumably caused by a decrease in the ability of maturation ameloblasts to remain attached to the enamel surface.

In bullous pemphigoid, autoantibodies are directed against BPAG-1 and BPAG-2, both components of hemidesmosomal plaques (see Fig 4-33). Bullous pemphigoid antigen 1 is a peripheral membrane protein localized along the inner leaflet of the plasma membrane, and BPAG-2 is a transmembrane protein. Association of autoantibodies to these proteins leads to the detachment of keratinocytes and formation of subepidermal blisters.

Pemphigus vulgaris is caused by autoantibodies to desmoglein 3.¹⁸² Pemphigus foliaceus is caused by autoantibodies to desmoglein 1. Both conditions lead to desmosomal disruption and blister formation in skin and oral mucous membranes.¹⁸³

Another disease of skin and mucous membranes resulting from destruction of the basement membrane attachment apparatus is lichen planus. This condition is characterized by disruption of the normal architectural association of collagen VII, integrin ($\alpha 6 \beta 4$), and anchoring filaments.¹⁸⁴ The cause is localized and chronic inflammation, initiated and maintained by an immune response to an unknown antigen. Increased expression and activity of matrix metalloproteinase 2 in lichen planus is believed to result in basement membrane degradation.¹⁸⁵ The subepithelial connective tissue is densely infiltrated by lymphocytes, and the basal cells show signs of degeneration.

Squamous cell malignant transformation

Unregulated cell proliferation leads to benign tumors or malignant neoplasms. Benign tumors are characteristically slow growing and contained. Nonmalignant tumor cells usually appear rather well differentiated and they often continue to express many of the same products found in normal cells.

Malignant neoplasms, or cancers, grow rapidly and aggressively. They invade adjacent tissues and spread to distant organs via lymphatics and/or blood vessels (metastatic spread). Aggressively growing cancers destroy normal tissues by releasing proteolytic enzymes, which digest the extracellular matrix, and cytokines that activate other cell types such as osteoclasts and macrophages. Neoplastic cells also consume nutrients that otherwise would be used in maintaining normal cells. Malignant cancer cells are

poorly differentiated, failing to produce products typically found in their cells of origin.

Squamous cell carcinoma, a malignant transformation of epithelial cells, is the most common cancer of the oral cavity, accounting for more than 90% of all oral cancerous lesions. It is an aggressive lesion that undergoes metastatic spread. It kills thousands of people annually. Men are four times more likely to develop the disease than women. Although the exact causes are still unknown, it is clear that smoking, tobacco chewing, and excessive alcohol consumption are among the determining factors.

Malignant transformation is a multistage process involving initiation, clonal expansion, and growth autonomy. The initiating step, caused by chemical, physical, or viral carcinogen, results in genetic damage (mutation). Cells with the mutated gene or genes undergo clonal expansion, stimulated by tumor promoter substances. The final and most time-dependent step in malignant transformation is the acquisition of growth autonomy.

An example of the multistage, or multiple genetic hits, model of cancer formation is the development of skin cancer. Sunlight increases the size and number of clones of *p53*-mutated keratinocytes.¹⁸⁶ Cells with defective *p53* genes are predisposed to undergo cancerous development on additional genetic damage. The role of *p53* in normal cells is to control cell proliferation by preventing cells from entering the G1 phase of the cell cycle (see chapter 13).

Mutations in the *p53* gene have been correlated to the development of several types of cancer.¹⁸⁷ The expression of *p53* is increased in hyperplastic lesions and squamous cell carcinomas of the oral mucosa.¹⁸⁸⁻¹⁹⁰ The increased presence of *p53* is believed to be the result of its mutated and more stable form. It has also been shown that p27, a cyclin-dependent kinase inhibitor, is decreased in cells of oral squamous cell carcinoma.¹⁹¹

In preparation for cell division, DNA and the mitotic machinery are checked for defects. If either of these components proves to be defective, cell division is stopped at either the DNA or the mitotic spindle checkpoints, and the cell enters a programmed cell death pathway (apoptosis). Failure of the normal operation of checkpoint surveillance and/or apoptosis usually leads to cancerous growth. There is evidence that the apoptosis surveillance system may malfunction during the development of SCC. Studies of premalignant and malignant SCC lesions indicate that the rate of cell proliferation increases more rapidly than does the rate of apoptosis.¹⁹²

Transforming growth factor β is a potent inhibitor of normal keratinocyte proliferation. A growing body of experimental evidence points to loss of TGF- β regulation as an early event in the conversion of benign epithelial tumors into malignant cancers (squamous carcinomas).¹⁹³ Genetic deletion of autocrine TGF- β in keratinocytes causes rapid transformation to squamous carcinoma.¹⁹³ Although the causes of malignant conversion of squamous epithelia are certainly complex, and many of the mechanisms have yet to be identified, a role for TGF- β seems certain. Neoplastic epithelial cells may fail to express TGF- β or fail to bind and activate autocrine and paracrine TGF- β , or some component in the signal transduction linkage between TGF- β and the cell-cycle regulatory proteins may be defective.

A loss, or abnormal expression, of integrins ($\alpha 6 \beta 4$, $\alpha 2 \beta 1$, and $\alpha 3 \beta 1$) in basal cells is a characteristic of oral squamous cell carcinomas.^{194,195} However the *de novo* expression of $\alpha \nu \beta 6$ in epithelial cells at the leading edge of SCC tumors suggests that adhesive contacts between $\alpha \nu \beta 6$ and tenascin promote tumor invasiveness.^{196,197} In vitro studies indicate that the addition of $\alpha \nu \beta 6$ to SCC cells decreases anchorage-independent growth while stimulating cell differentiation.¹⁹⁴

An additional defect observed in some human squamous cell carcinoma cell lines (including buccal mucosa and tongue) is that of a decreased ability to form gap junctions.^{198,199} Neoplastic cells may lack growth control regulated by cell-to-cell communication with normal cells.²⁰⁰ Decreased gap junctional communication could be the result of reduced expression of connexin molecules or the inability of the tumor cells to adhere to normal cells long enough for gap junction formation. Both gap junctions and desmosomes are reduced by more than 50% in chemically induced SCC.²⁰¹ Decreased frequency of hemidesmosomes was also reported in SCC.²⁰² Of significance in this regard is the recent report that some tumor-promoting substances inhibit gap junctional communication and the normal display of E cadherins.²⁰³

Evidence of decreased expression of desmogleins, desmoplakins, and E cadherins has been reported in squamous cell carcinomas of the mouth.^{204,205} A direct correlation between the loss of desmosomal proteins and tumor metastasis has been observed in SCC.²⁰⁶ In vitro experiments have demonstrated that invasive cells transfected with desmosomal proteins lose their ability to migrate into collagen gels.²⁰⁷ These results support the contention that desmosome formation suppresses the spread of cancer cells. Decreased expression of syn-

decan 1 is also directly related to the invasiveness of SCC.¹⁵¹

Vitamin A metabolites and their receptors participate in the regulation of keratinocyte proliferation and differentiation. Although the mechanisms of action remain elusive, there have been numerous reports of the antiproliferative action of retinoids in SCC cells.²⁰⁸ In some cases, retinoids have been used as chemopreventive agents to reverse premalignant lesions and to prevent the development of secondary tumors.^{209,210}

The search to identify the molecular mechanisms responsible for transforming a normal cell into an unregulated cancer cell is one of humankind's greatest challenges. With each year, as new findings are reported on the control of gene transcription, signal transduction, growth factor interaction, and the regulation of cell adhesion and cell migration, progress is made in understanding the nature of cancer cells. The aforementioned examples are but a few of the many to be found in the current research literature.

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Chapter 5

Gingiva



The gingiva is that portion of the oral mucosa that covers the tooth-bearing part of the alveolar bone and the cervical neck of the tooth (Fig 5-1). The structural and physiologic characteristics of the gingiva have been described extensively by Schroeder¹ and Schroeder and Listgarten.^{2,3}

The gingival epithelium is relatively thick and well cornified on its oral surface but thin and noncornified as it is reflected back to form the lining of the gingival sulcus and the junctional epithelial attachment (see Fig 5-1). Except for a narrow zone of free gingiva, defined coronally by the gingival margin and apically by an imaginary horizontal line drawn to the base of the junctional epithelium (JE), the bulk of the gingiva is firmly attached to the tooth and the alveolar bone by well-developed collagenous fiber bundles. The free gingiva is a relatively mobile tissue surrounding the gingival sulcus. It covers approximately 1.0 to 1.5 mm of the tooth surface (see Fig 5-1).⁴ The width of the keratinized gingiva (attached gingiva plus the free gingiva) may vary from 1.0 mm to 9.0 mm.⁵

The apical boundary of the attached gingiva is located at the mucogingival junction, where the cornified epithelium merges abruptly with the noncornified epithelium of the alveolar mucosa.⁶ The mucogingival junction is a stable landmark, probably genetically predetermined.⁷ The width of attached gingiva varies for each tooth. In general, the attached gingiva is wider in the maxilla, especially on the labial surfaces of the incisors, and narrowest over the buc-

cal surfaces of the mandibular canines and first premolars and the lingual surfaces of the mandibular incisors.^{5,8,9} The width of the attached gingiva varies from 1.0 to 6.0 mm.¹

The mean width of attached gingiva increases from the primary to the adult dentition.⁹ The anatomic width of the attached gingiva increases slightly with increased age, as the teeth erupt, to compensate for occlusal wear.⁷ When the width of the attached gingiva falls below 1.0 mm (2.0 mm if the free gingiva is included), the risk of developing gingival and periodontal disease is greater unless strict oral hygiene is practiced.^{1,5}

In about one third of all individuals, a shallow free gingival groove runs parallel to the gingival margin along a line that is located roughly at the junction between the free gingiva and the attached gingiva.⁴ Because the free gingival groove is not a constant anatomic feature, it must not be considered a marker of gingival health.⁴ It may or may not be present, irrespective of the level of gingival inflammation.

Another anatomic feature that is variably present is stippling. Stipples are small, regularly spaced depressions in the surface of the attached gingiva that give it an "orange peel" appearance.^{10,11} The absence of stippling is not a sign of disease, and conversely its presence is not necessarily a sign of gingival health.¹²

Depending on the anatomic configuration of the interproximal area between adjacent teeth, and the

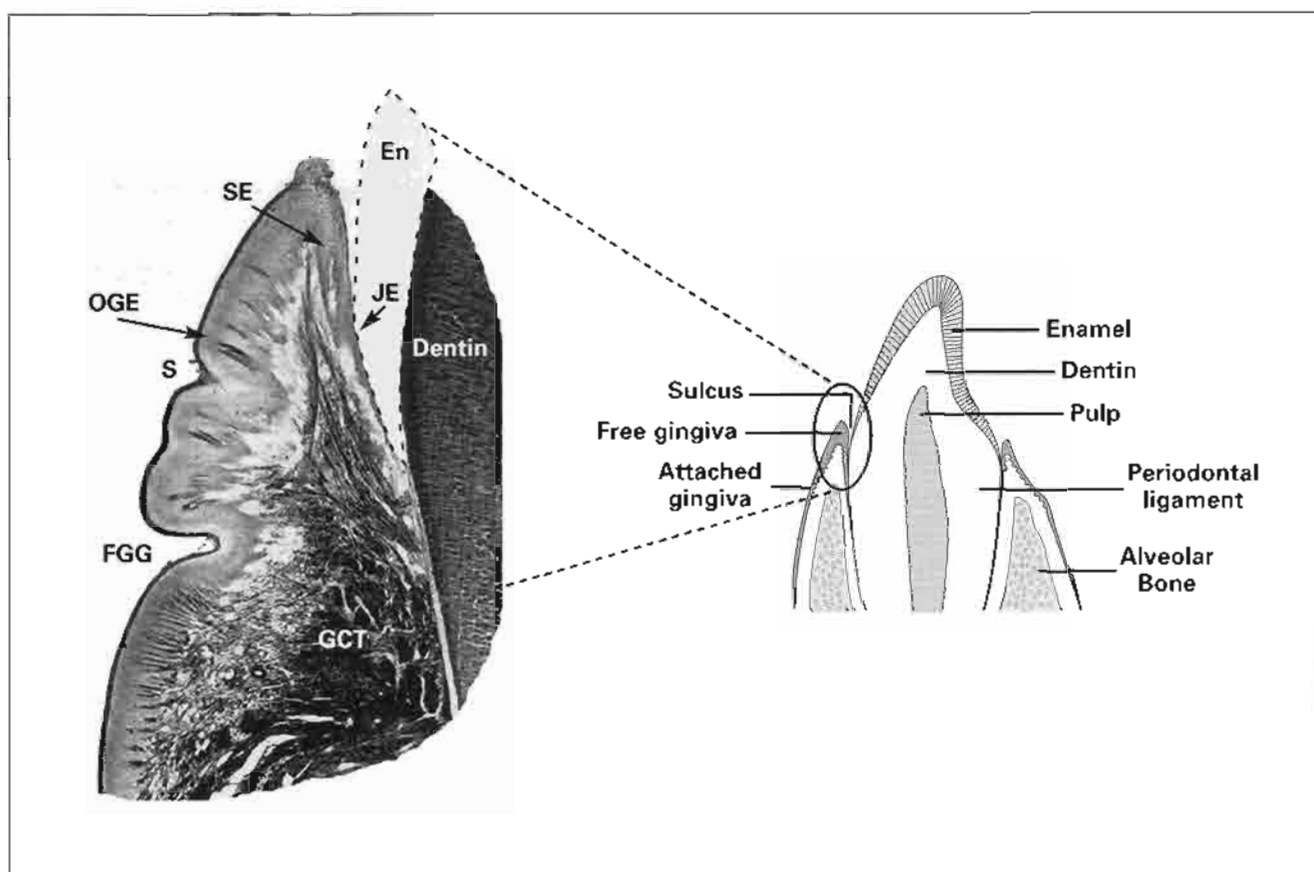


Fig 5-1 Gingival tissues and their relationship to other parts of the tooth and periodontium. (En) Enamel space; (FGG) free gingival groove; (GCT) gingival connective tissue; (JE) junctional epithelium; (OGE) oral gingival epithelium; (S) stipple; (SE) sulcular epithelium.

size and location of the contact area, the interdental gingiva assumes the shape of a dental papilla or that of a col, a concave dip in the epithelium beneath the contact area.¹³ The epithelial lining of the col is of a similar type to that of the JE and is contiguous with it.

The biologic significance of the topographic subdivision of the gingival tissues into free, attached, and interdental gingiva has been challenged.³ It is true that less emphasis should be placed on minor anatomic variations and more attention paid to the endogenous and exogenous factors, which regulate the integrity of the collagenous matrix and the permeability of the overlying epithelial barriers. The focus of today's research must be to understand how the body's defensive systems adapt, or fail to adapt, to the constant presence of bacteria at the tooth-gingival interface and to obtain the technology

to reverse the effects of disease and to regenerate lost tissues.

The epithelial components of gingiva represent a continuum from thick, impermeable oral gingival epithelium (OGE) to the highly permeable junctional epithelium. The oral sulcular epithelium (OSE) represents a transitional area of variable length, depending on the level of underlying inflammation. Generally the transition between the OGE and the OSE occurs close to the crest of the gingiva, while the junction between the OSE and the JE occurs on a diagonal line so that a portion of the OSE overlaps the JE. The most coronal cells of the JE form the base of the gingival sulcus. Differences in keratinocyte differentiation, epithelial permeability, and innervation characterize each of these regions. The biologically significant differences in these three domains form the basis for the following discussion.

Epithelial Components of the Gingiva

Oral gingival epithelium

The OGE is cornified, impermeable to water-soluble substances, and attached firmly to a base of dense gingival connective tissue. Four clearly defined cell layers are present: the basal cell layer, the spinous cell layer, the granular cell layer, and the cornified cell layer. The basal cells make up the proliferation compartment of the epithelium and the remaining layers form the differentiation compartment.

Oral gingival epithelial cells have the lowest rate of proliferation in comparison to JE and OSE cells. It has been suggested that this may be due to the physiologic restricting effect of transforming growth factor β (TGF- β) on epithelial cell proliferation. The cells of the OGE have been shown to express higher levels of TGF- β and its receptors than the cells of the OSE and JE.¹⁴

There is a high degree of interdigitation (rete peg formation) between the OGE and the underlying connective tissue.¹⁵ Contact between the two tissues is further amplified by the presence of numerous serrated keratinocytes and the formation of prominent cell processes (pedicles) that protrude into the connective tissue (Fig 5-2). Basal cells attach to the lamina densa of the basal lamina through the formation of many hemidesmosomes. Anchoring fibrils made of type VII collagen bind the lamina densa to type I and type III collagen fibrils (see Fig 5-2).¹⁶ The structure and molecular composition of the components of the cell membrane and extracellular matrix that form this attachment complex are discussed in chapter 4.

Spinous layer cells of the OGE are specialized for cell-to-cell contact via their many desmosomes. These cells contain many keratin filament bundles (tonofibrils) that associate peripherally with the attachment plaques of desmosomes (Figs 5-3a and 5-3b). The number of desmosomes per cell doubles from the basal to the spinous layer.⁶ Cell contacts of the gap junction variety are also abundant. Nonkeratinocytes located in the OGE include melanocytes, Langerhans cells, and Merkel cells.^{6,17,18}

Similar to the differentiation pattern found in skin, the stratum granulosum of the OGE contains membrane-coating granules, keratohyalin granules, and numerous tonofibrils. The transition to the stratum corneum is abrupt (see Fig 5-3b). The flattened cornified squames form a relatively thick protective cover-

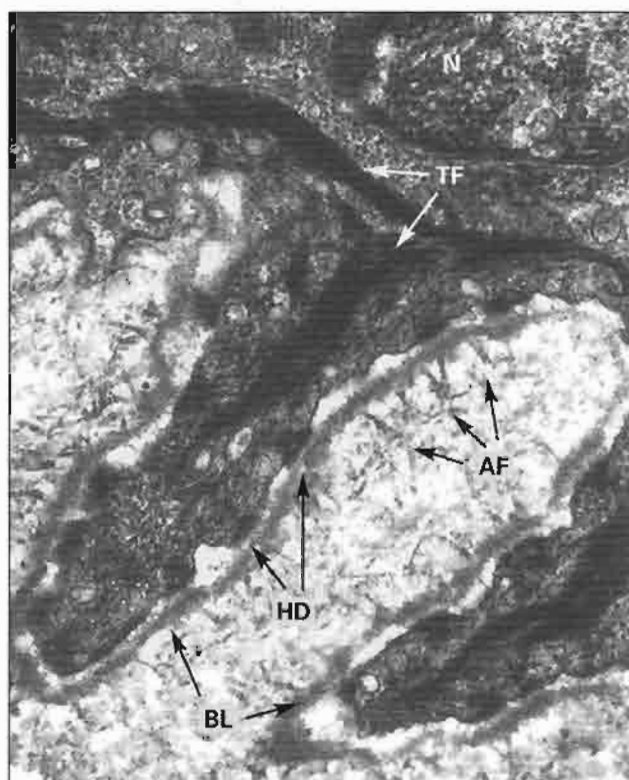


Fig 5-2 Cell processes protrude from basal cell into the connective tissue, forming a strong bond to the underlying type I and type III collagen fibrils via anchoring fibrils (AF), the lamina densa of the basal lamina (BL), and the hemidesmosomes (HD). (N) Nucleus; (TF) tonofibrils. (Original magnification $\times 21,000$.)

ing over the connective tissue and the epithelial attachment.

Parakeratinization, a condition characterized by incomplete disintegration of the nucleus and cytoplasmic organelles, is usually observed in the stratum corneum of the OGE.⁶ However, orthokeratinization, the complete digestion of the nucleus and organelles accompanied by a more complete and uniform cornification, as found in skin, may also occur in OGE. Examination of diseased tissue suggests that inflammation of the underlying connective tissue is partly responsible for the incomplete cornification of the OGE.

Further support for the influence of connective tissue in determining the differentiation pathway of oral epithelium was obtained in transplantation experiments. These studies showed that the OGE connective tissue can alter the differentiation of the epithelial cells of the alveolar mucosa, a tissue that does not normally undergo cornification. When alveolar

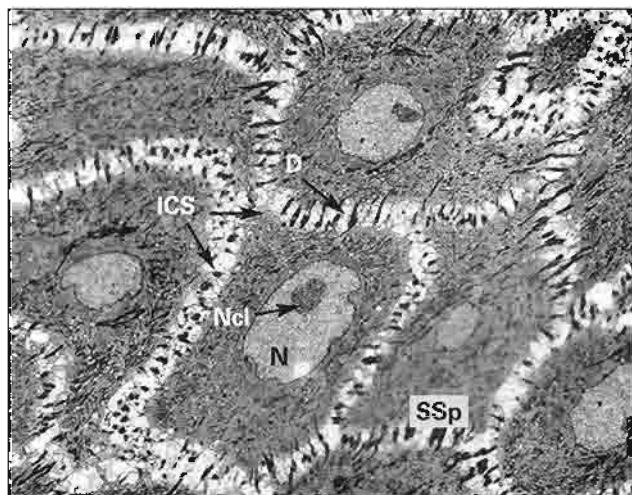


Fig 5-3a Cells of the stratum spinosum (SSp) of the OGE are characterized by large euchromatic nuclei (N) with prominent nucleoli (Ncl), and numerous desmosomes (D) along the intercellular space (ICS). (Original magnification $\times 2,200$.)

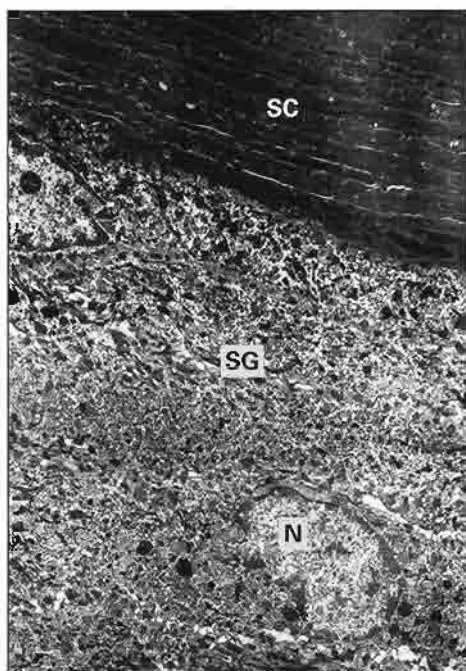


Fig 5-3b Abrupt change from the stratum granulosum (SG) to the stratum corneum (SC) with its densely packed keratin filaments. (N) Nucleus. (Original magnification $\times 2,200$.)

mucosa is grown over gingival connective tissue, it becomes cornified.¹⁹

In summary, the OGE:

1. Amplifies the interface between basal cells and connective tissue, to provide a firm attachment for the epithelium.
2. Has a high level of differentiation of spinous cells to form numerous keratin fibrils and desmosomes, serving to increase the stability of the epithelium.
3. Develops a permeability barrier to water-soluble substances.
4. Forms a cornified protective outer layer.

Oral sulcular epithelium

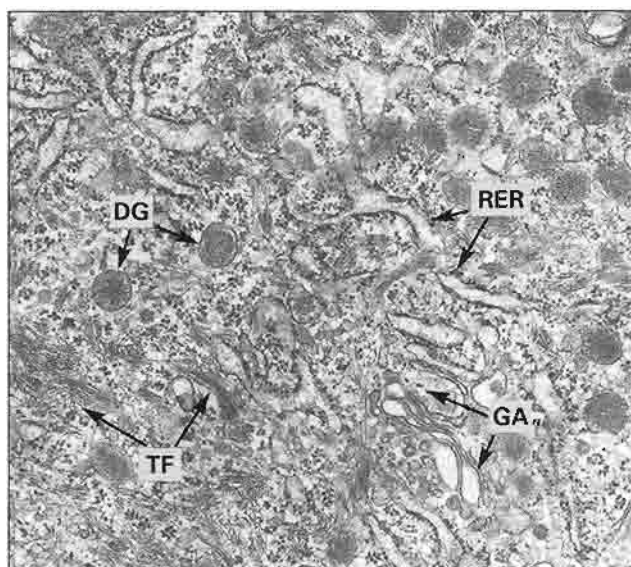
The OSE extends apically from the crest of the marginal gingiva to the JE (see Fig 5-1). Regular and prolonged chemical and mechanical toothcleaning can lead to a condition in which there is almost no sulcus. In this superhealthy state, achieved under experimental conditions, the JE extends along the enamel up to the gingival margin, and the underlying connective tissue is free of inflammatory infiltrates.²⁰ However, most individuals who practice good oral hy-

giene have clinically healthy gingiva. This condition is characterized by a shallow gingival sulcus (less than 3.0 mm), no bleeding on probing, moderate numbers of inflammatory cells in the connective tissue and JE, a small loss of collagen matrix beneath the OSE and JE, and a minimal flow of gingival fluid.

Deep interdigitations of basal cell podocytes and connective tissue, as observed in OGE, are not as well developed beneath the OSE. Although the OSE is stratified, it does not contain a clearly defined stratum granulosum, nor does it normally undergo cornification.^{21,22} Extirpation experiments, in which the sulcular epithelium was removed and grown on a bed of healthy connective tissue, demonstrated that it could undergo cornification. This observation suggests that the degree of inflammation in the underlying gingival connective tissue may have a regulatory influence over the level of cornification attained by the OSE.

The differentiating compartment of OSE contains inner and outer zones. The inner zone resembles a spinous layer, but individual cells contain fewer tonofibrils and desmosomes than do cells in the spinous layer of the OGE.¹ The cells of the inner zone tend to be flattened and to lie parallel to the epithelial surface. The outer zone contains viable cells with in-

Fig 5-4 Cytoplasm of a keratinocyte in the outer layer of the oral sulcular epithelium. Numerous dense granules (DG) are present. The cisternae of the Golgi apparatus (GA) and the rough endoplasmic reticulum (RER) are fairly well developed in these cells. (TF) Tonofibrils. (Original magnification $\times 14,000$.)



tact nuclei and abundant cytoplasmic organelles. The superficial cells, which are shed into the sulcus, demonstrate considerable variation in shape and density; some are thin and darkly basophilic, while others are large and lightly stained.^{21,22} Different degrees of hydration and plasma membrane integrity may account for these differences.

Keratin filaments are bundled into small tonofibrils that are loosely distributed throughout the cytoplasm.²¹ Keratohyalin granules and membrane-coating granules are rarely observed in cells of the OSE. The outermost cells contain a moderate amount of rough endoplasmic reticulum, Golgi membranes, and membrane-bound dense granules (Fig 5-4).²¹ Although the nature of the dense granules has not been satisfactorily established, some reports suggest that they belong to the lysosomal system, while other reports have indicated that they might be a variant of the membrane-coating granule.²³

In inflamed gingiva, the OSE is infiltrated by numerous polymorphonuclear neutrophil leukocytes (PMNs) and lymphocytes.^{24,25} Changes brought about by the infiltrating inflammatory cells include a loss of desmosomes and a widening of the intercellular spaces. When viewed in electron microscopes, the intercellular spaces of inflamed gingiva contain a fine precipitate that is believed to be of serum origin.^{24,25}

In summary, the OSE:

1. Does not contain keratohyalin granules.
2. Is normally noncornified.

3. Is more permeable to water-soluble substances than is the OGE.
4. Contains increased lysosomal activity.

Junctional epithelium

Junctional epithelial cells maintain a direct attachment to the tooth surface. The earliest attachment forms following completion of enamel maturation, when the reduced ameloblasts attach to the enamel via hemidesmosomes and a granular basal lamina-like material (the internal basal lamina). No cuticular matrix is present between the basal lamina and the enamel.²⁶ In classic histology, this first attachment is termed the *primary epithelial attachment*.

During eruption, contact is established between the reduced enamel epithelium and the oral gingival epithelium. Mitotic activity increases in the reduced enamel epithelium, and changes in cell shape and organelle content take place as the cells merge with the oral mucosa to form a JE. This process gives rise to the *secondary epithelial attachment*.²⁷

The basal cells of the JE are separated from the connective tissue by the external basal lamina (Fig 5-5). The interface between the JE and the underlying connective tissue is relatively smooth, unlike the condition found in the OGE. Epithelial rete peg formation from the JE (and the OSE) is a condition found only in highly inflamed connective tissue.

Although JE does not exhibit true phenotypic stratification, the outermost cells tend to be elongated and to lie with their long axis parallel to the

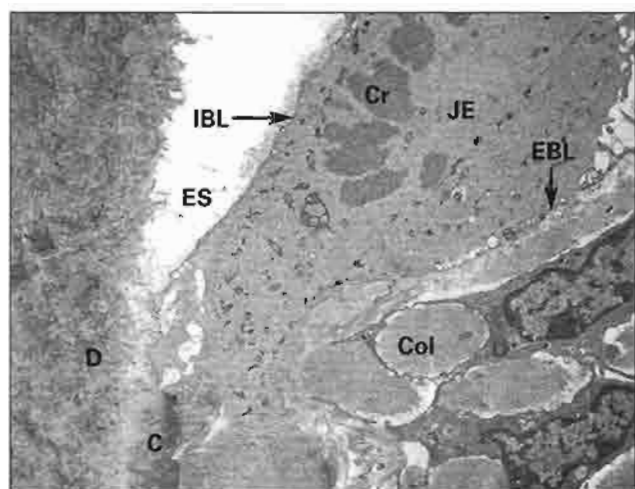
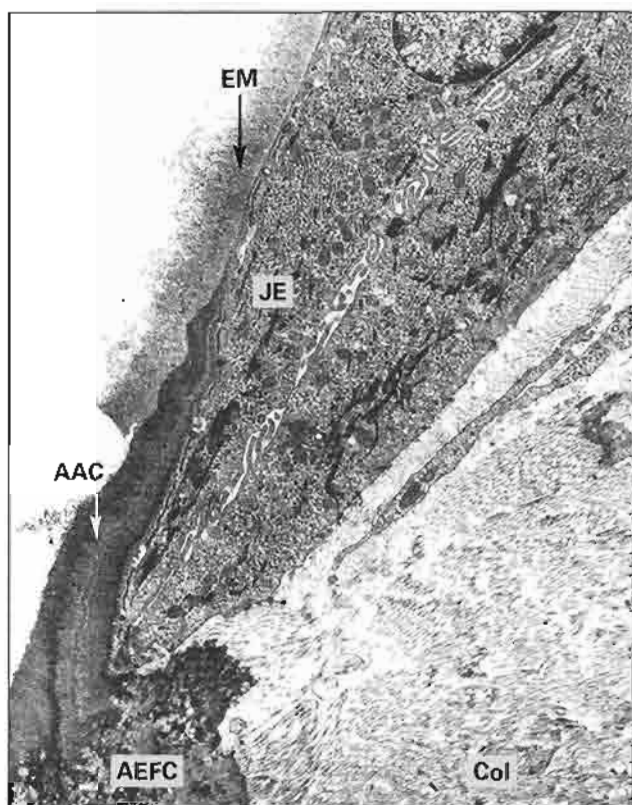


Fig 5-5 Mitosis in the most apical cell of the junctional epithelium (JE). (C) Cementum; (Col) collagen bundle; (Cr) chromatin; (D) dentin; (EBL) external basal lamina; (ES) enamel space; (IBL) internal basal lamina. (Original magnification $\times 4,000$.)

Fig 5-6 Apical end of the junctional epithelium (JE), attached to a spur of afibrillar acellular cementum (AAC) or type A cuticle. (AEFC) Acellular extrinsic fiber cementum; (Col) collagen fibers; (EM) enamel matrix. (Original magnification $\times 6,100$.)



tooth surface.³ The number of desmosomes is significantly reduced in relation to the number found in the OGE.^{1,28} Suprabasal cells of the JE express keratin markers typically found in basal cells and simple epithelia. The JE tapers from its coronal end, which may be 15 to 30 cells wide, to one or three cells at its apical termination, located at the cemento-enamel junction in healthy tissue (Figs 5-5 and 5-6).⁶

At the tooth surface, the outermost epithelial cells of the JE produce an internal basal lamina and are anchored to this basal lamina by numerous hemidesmosomes.^{3,29,30} Keratin tonofilaments are not inserted into the hemidesmosomes along the internal basal lamina (Fig 5-7). The internal basal lamina is approximately three times thicker than the external basal lamina.³¹ The internal basal lamina contains glycoproteins, laminin, and proteoglycans.^{32,33} Attempts to localize collagen type IV in this structure have been unsuccessful.³⁴

Cells in contact with the internal basal lamina express the $\alpha 6 \beta 4$ integrin, a laminin receptor.³⁵ The cells in contact with the internal basal lamina contain a relatively well-developed rough endoplasmic reticulum and numerous Golgi components.^{36,37} Studies of cellular utilization of tritiated thymidine indicate

that the cells in contact with the tooth surface are capable of proliferation and thus could contribute to regeneration of the JE.

The external basal lamina contains collagen type IV, heparan sulfate proteoglycan, laminin, and fibronectin.³² Anchoring fibrils are less prominent in the connective tissue below the JE than they are in the OGE.

Electron microscopic cytochemical studies have shown that the cells of the JE contain a moderately well-developed lysosomal system and participate in the phagocytosis of material from the intercellular space.³⁸⁻⁴⁰ When tracer molecules, such as horseradish peroxidase and concanavalin A, are injected intravenously or applied topically to the sulcus, they gain access to the intercellular space of the JE and are taken up in endocytotic vesicles by the cells of the JE.^{39,41-43} Cathepsins (B, D, and H) and acid phosphatase, both indicative of degradative enzyme activity, have been localized to primary and secondary lysosomal structures in the cells of the JE. Several investigators have concluded that the endocytic capacity of the cells of the JE is equal to that of macrophages and neutrophils and that this activity might serve a protective function. The cells of the JE

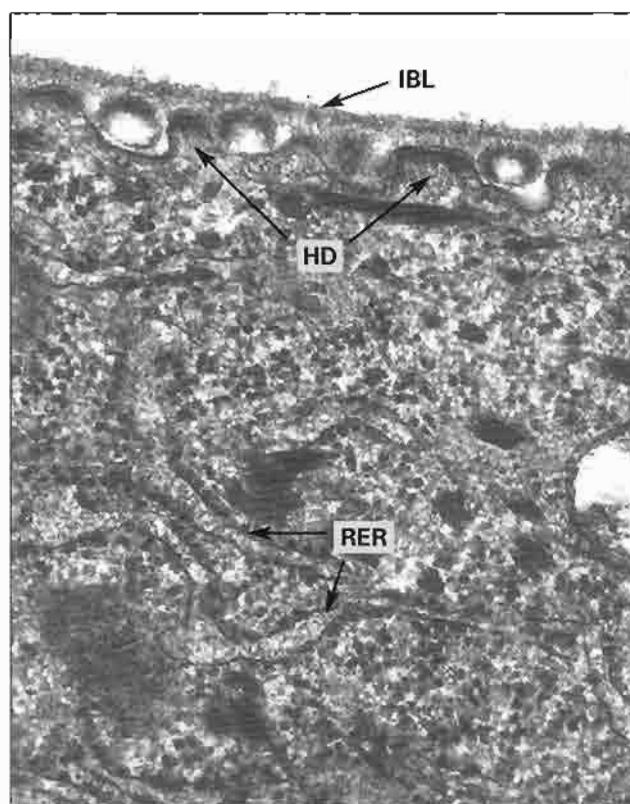


Fig 5-7 Electron micrograph of the internal basal lamina (IBL) between tooth enamel (not shown because of the demineralization of the section) and the junctional epithelium. Note the many hemidesmosomes (HD) and cisternae of rough endoplasmic reticulum (RER). (Original magnification $\times 29,000$.)

may also contribute lysosomal cathepsins to the gingival crevicular fluid (GCF).

Characteristically, the JE has wide intercellular spaces, and the keratinocytes exhibit numerous cytoplasmic folds that extend into the intercellular space.¹ Cells of the JE show no signs of synthesis of membrane-coating granules, a finding that agrees with the fact that the JE is highly permeable to water-soluble substances.⁴⁴ The chief barrier to passage of substances larger than 100 kDa is provided by the external basal lamina. Because of the absence of an effective permeability barrier among the cells of the JE, it provides an open pathway for the penetration of bacterial antigens, lipopolysaccharides (LPSs), and enzymes from the sulcus to the connective tissue.^{42,43,45-47} Sulcular fluid, a protein-rich fluid derived from transudation of serum and extracellular fluid, flows in an outward direction through the JE.⁴⁶ This fluid contains antibodies, complement, and enzymes that form an antibacterial defense system.

The penetration of antigens from the gingival sulcus into adjacent connective tissue and local draining lymph nodes has been studied in dogs and rodents; horseradish peroxidase was used as the immunizing antigen.^{45,48} Because the reaction products of horseradish peroxidase could be readily visualized in the electron microscope, it was possible to trace horseradish peroxidase antigen in the JE, gingival connective tissue, and cervical lymph nodes at various times after a challenge dose was applied to the coronal area of the gingival sulcus. Within minutes, the antigen gained access to the intercellular spaces of the JE, and by 1 hour it was cleared from the gingival connective tissue by macrophages and lymphatics. Within 3 to 5 days, mature plasma cells producing anti-horseradish peroxidase antibodies were present in the germinal centers of the cervical lymph nodes.

The JE also serves as the primary pathway for transmigration of PMNs into the sulcus.^{3,36} Expression of cell surface attachment molecules by the cells of the JE, and the underlying endothelium, have been shown to promote PMN transmigration across the JE.⁴⁹ The widened intercellular spaces that are typically observed in electron micrographs of the JE are not artifacts created during tissue preservation. It has been suggested that the widened intercellular spaces of the JE provide the avenue for the transmigration of polymorphonuclear leukocytes and the exudation of crevicular fluid.³

Although the JE contains fewer cell-to-cell junctions per equivalent length of plasma membrane than does the OGE, there are many well-developed gap junctions and desmosomes. Small adherens junctions are also present. In general, there appears to be an inverse relationship between the numbers of infiltrating PMNs and epithelial cell-to-cell junctions.

In the interdental spaces, the gingival epithelium dips apically from a vestibular to an oral direction, forming a depression, or col, beneath the contact point. This col epithelium shares the same structural characteristics as the JE.

The JE has a high rate of turnover.^{50,51} Daughter cells are produced along the external basal lamina. Cells leave the external basal lamina, migrate to the free surface of the JE located at the base of the gingival sulcus, and are exfoliated. As measured in non-human primates, the rate of cell turnover in the JE is approximately 5 to 10 days, faster than that observed in the OSE and OGE.⁵⁰

Mechanical separation of the JE from the tooth surface and its subsequent replacement (as in a surgical flap procedure) leads to regeneration of the epithelial attachment in about 7 days, accomplished by

the proliferation and migration of basal cells from the most apical part of the JE. Studies have shown that, following mechanical separation of the JE from the tooth, some junctional epithelial cells remain in contact with the tooth surface and that can proliferate to regenerate the JE attachment.⁵²

Following surgical removal of the entire gingiva, a new junctional epithelium forms from adjacent oral epithelium by migration of cells from the cut epithelial edge toward the tooth surface.⁵³ At least 2 weeks are needed for regeneration of a complete JE. This new JE will extend apically until it encounters a firm collagen fiber attachment to cementum.

Differences in cell proliferation rates among the three regions of gingival epithelium may be the result of their responsiveness to the growth-inhibitory effect of TGF- β . The cells of OGE express receptors for TGF- β , while the more rapidly proliferating cells of the JE have fewer TGF- β receptors.

Another factor that may account for different proliferation rates is epidermal growth factor (EGF). Cells of healthy JE contain high levels of epidermal growth factor (EGF) and express EGF receptors.^{54,55} Under the same conditions, the OGE and the OSE are negative for EGF and EGF receptor. It has been observed that EGF receptor increases in the OGE and OSE during inflammation.⁵⁶ Tumor necrosis factor (TNF), a cytokine present in inflammatory tissue, is a stimulator of EGF receptor expression. Epidermal growth factor receptor is an integral membrane protein with a long intracellular domain that contains tyrosine kinase activity. Activation of the receptor by EGF leads to phosphorylation of the receptor and other intracytoplasmic proteins, culminating in increased DNA and protein synthesis and increased cell motility.

In healthy teeth, which have not had any prior loss of attachment, the JE (epithelial attachment) ends at the cemento-enamel junction (see Figs 5-5 and 5-6). Densely packed collagen bundles are anchored to the acellular extrinsic fiber cementum just below the terminal point of the JE. These collagen bundles form the connective tissue attachment. The stability of this connective tissue attachment is a key factor in limiting the apical migration of the JE.

Resorption of collagen along the root surface beneath the JE removes a barrier to epithelial migration. Collagenolytic enzymes involved in the destruction of the connective tissue attachment may originate from fibroblasts, macrophages, and neutrophils located next to the JE. Of significance is the finding that keratinocytes can produce collagenolytic enzymes when stimulated by cytokines. It has also been reported

that interleukin 1 (IL-1) produced by epithelial cells stimulates the secretion of collagenase by fibroblasts.

In inflamed gingiva, the epithelial cells at the leading edge of a migrating JE have no internal or external basal lamina and no hemidesmosomes. They resemble the epithelial cells observed at a wound edge. At a distance apical to the leading edge, the epithelial cells attach to mineralized cementum by developing a basal lamina and forming numerous hemidesmosomes. This attachment appears similar to that formed on enamel.

In summary, the JE:

1. Has a high rate of proliferation.
2. Is noncornified and poorly stratified.
3. Is highly permeable.
4. Is the main passageway for neutrophil entry into the gingival sulcus.

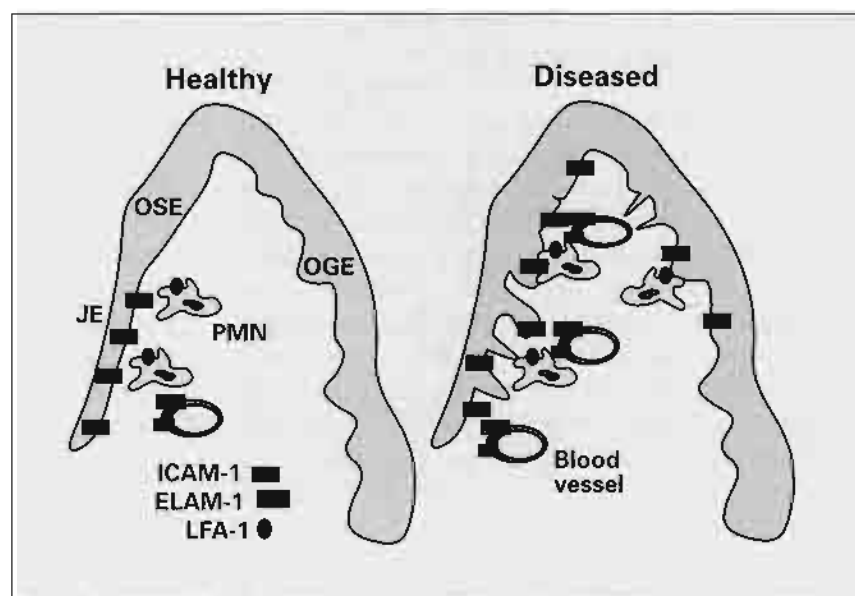
Expression of Keratins in Gingiva

Keratins (Ks) form a family of some 20 polypeptides, divided into acidic and basic subfamilies. The molecular structure of keratin is described in chapter 4. The basic to neutral keratins are numbered K1 to K8, and the smaller and acidic keratins are numbered K9 to K19. Because keratin fibrillogenesis requires the assembly of pairs of acidic and basic polypeptides, keratin molecules are usually expressed in pairs.

In general, K5 and K14 are expressed in all stratifying epithelia. Keratin 1, K2, K10, and K11 are expressed in the suprabasal cells of cornifying epithelia, while K4 and K13 are expressed in the suprabasal cells of noncornifying (and parakeratinized) stratifying epithelia.⁵⁷ Both K6 and K16 are usually expressed in hyperproliferative epithelia. Types K7, K8, K18, and K19 are expressed by simple epithelia and are useful markers for the localization of epithelial neuroendocrine cells (Merkel cells).

The availability of antibodies to specific keratins has made it possible to study the variation in keratin expression in various oral epithelial tissues as well as in the three separate areas of the gingival epithelium.⁵⁷ Application of immunofluorescent antikeratins to the OGE has shown that it stains for K5, K14, K1, K2, K10, K11, K6, K8, K16, K18, and K19 in isolated cells.⁵⁸ The OSE stains for K5, K14, K4, K13, K6, K16, and K19. The JE expresses K5, K14, K13, and K19. Although the JE has a high rate of turnover, there is conflicting data on the presence of K6 and K16 in that location. The lack of K4 in JE marks the boundary between it and the OSE. The boundary be-

Fig 5-8 Adhesion molecules and polymorphonuclear neutrophil (PMN) transmigration. In healthy gingiva, intercellular adhesion molecule 1 (ICAM-1) is expressed by cells of the junctional epithelium (JE), and endothelial leukocyte adhesion molecule 1 (ELAM-1) is expressed on endothelial cells beneath the JE. Neutrophils express leukocyte function antigen 1 (LFA-1), a binding ligand for ICAM-1 and ELAM-1. Cell-to-cell attachments via these adhesion molecules are important in PMN infiltration of the JE and sulcus. With increased inflammation, the expression of ICAM-1 and ELAM-1 spreads to other regions of the gingiva. (OGE) Oral gingival epithelium; (OSE) oral sulcular epithelium.



tween OSE and OGE is not sharply defined by keratin types, because there is some overlap of the suprabasal cell types along the crest of the gingiva.

The local factors that regulate the spectrum of keratin gene expression in the three gingival zones are undoubtedly complex. Recent *in vitro* studies indicate that the metabolism of retinoic acid may play a role in regulating keratin expression because it enhanced the expression of K8 and K18 in OGE,⁵⁹ but decreased K1 and filaggrin.⁶⁰

The K4/K13 pair of keratins is typically expressed by lining mucosa associated with the properties of flexibility and elasticity, whereas the K1/K10 pair expressed by masticatory mucosa, hard palate, and skin is associated with rigidity and toughness.

Expression of Cell Surface Adhesion Molecules in Gingiva

Integrins are a large family of transmembrane glycoproteins, which serve to attach cells to a large number of extracellular matrix ligands such as fibronectin, laminin, vitronectin, tenascin, and osteopontin. The integrins are heterodimers formed by noncovalent association of α and β glycoprotein subunits (see chapter 1). In humans, more than 15 heterodimers are formed from at least 14 α chains and eight β chains.

Recent studies have demonstrated that basal cells and suprabasal cells of the JE, OSE, and OGE

express the integrins $\alpha 2\beta 1$, $\alpha 3\beta 1$, and $\alpha 6\beta 1$.⁶¹ The $\alpha 6\beta 1$ integrin is a component of desmosomes. Hemidesmosomes contain the $\alpha 6\beta 4$ integrin (laminin receptor) localized on both the internal and external basal laminae of the JE.³⁵ During gingival inflammation, the expression of integrins, especially those that function as fibronectin receptors, increases in cells of both the epithelial and connective tissues.⁶²

Calcium-binding surface adhesion molecules, the cadherins, are components of desmosomes and adherens junctions (see chapter 4).

Another class of cell surface adhesion molecules that is of significance to the biology of gingival tissues is the immunoglobulin class, of which intercellular adhesion molecule 1 (ICAM-1), endothelial leukocyte adhesion molecule 1 (ELAM-1), and vascular cell adhesion molecule 1 are known to increase in gingiva during inflammation.⁶³ Intercellular adhesion molecule 1 interacts with the leukocyte function-associated antigen 1, a $\beta 2$ -type integrin expressed on leukocytes. Binding of leukocyte function antigen 1 to ICAM-1 appears necessary for normal transmigration of neutrophils through epithelia and for the migration of T lymphocytes into epithelial tissues.⁶⁴

Intercellular adhesion molecule 1 is present on the cell membrane of JE cells and adjacent fibroblasts and endothelial cells but absent from healthy OSE and OGE and adjacent blood vessels (Fig 5-8).⁴⁹ Keratinocytes of OSE and OGE express ICAM-1 only when the adjacent connective tissue becomes in-

flamed. Endothelial cells in the inflamed connective tissue also are immunoreactive for ICAM-1.

There is evidence that ICAM-1 is downregulated in epithelia of chronic periodontal inflammatory lesions. However, ICAM-1 is not the only adhesion factor promoting leukocyte adhesion to epithelial tissues, for even in the absence of positive ICAM-1 immunoreactivity there are numerous leukocytes in inflamed gingival epithelia. Patients who have a genetic defect in the expression of the $\beta 2$ chain of the leukocyte function antigen 1 integrin molecule, however, suffer from leukocyte adhesion deficiency, a condition that makes them prone to severe infections, including prepubertal periodontitis.

Another adhesion factor important for leukocyte transmigration in inflammatory lesions is ELAM-1. Endothelial leukocyte adhesion molecule 1 has been localized on blood vessels of gingivitis lesions (see Fig 5-8).⁶⁵ This molecule is expressed on endothelial cells that have been activated by cytokines such as TNF- α , IL-1, and bacterial lipopolysaccharide (see "Primary proinflammatory cytokines and chemokines," later in this chapter). The role of integrins and other cell surface adhesion molecules in leukocyte exudation from blood vessels is discussed in chapters 13 and 14.

Formation of Dental Cuticles

Dental cuticles are formed by precipitation of various proteins on the tooth surface, usually along the cervical part of the crown, between the enamel and the junctional epithelium. Listgarten described and characterized two types of cuticular deposits on the enamel surface.⁶⁶ Type A cuticle, found on both erupted and unerupted teeth, has a granular matrix with appositional lines (see Fig 5-6). It is usually restricted to the cervical area around the cemento-enamel junction and can be approximately 1 to 5 μ m thick and up to hundreds of microns in length. Type A cuticle is mineralized. Because of its structure, location, and ability to mineralize, it is thought to be a form of afibrillar, acellular cementum.

Type B cuticle is found only in erupted teeth. It is located between the enamel (or a type A cuticle) and the internal basal lamina of the JE. Type B cuticles have no appositional lines and do not mineralize. The current hypothesis is that type B cuticles are formed by the precipitation of tissue fluid proteins on the enamel and/or cementum surface. When slices of enamel or dentin are exposed to fresh serum, a cuticle similar to type B is deposited.

Type B cuticles are not observed over the enamel of unerupted teeth, suggesting that the reduced enamel epithelium protects the enamel surface from contact with tissue fluids.

Gottlieb, a dental histopathologist of the early 20th century, described a "primary enamel cuticle" covering the enamel of unerupted teeth. He speculated that this material was the end product of ameloblastic activity. Listgarten was unable to confirm the presence of this structure and concluded that it was an optical artifact produced when thick ground sections were examined by light microscopy.²⁶

Organization of Gingival Connective Tissue

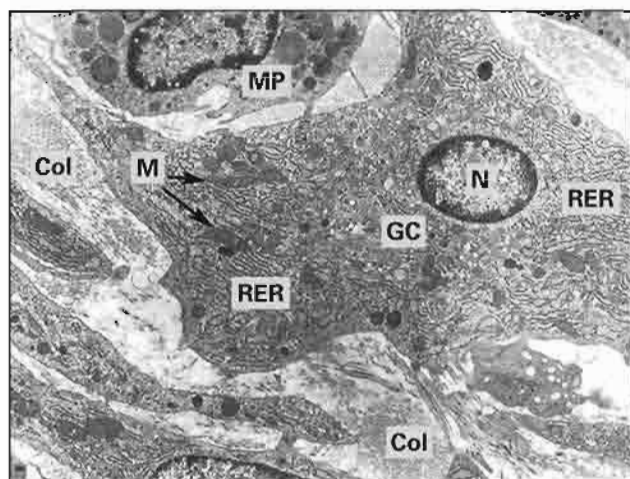
The collagen matrix of gingival connective tissue is well organized into fiber bundles, which constitute the gingival supra-alveolar fiber apparatus.¹ Transseptal, circular, semicircular, transgingival, and intergingival fibers connect and link the adjacent teeth of one arch. These fibers secure the teeth against rotation and maintain tooth linkage during mesial drift.

Tractional forces in the extracellular matrix produced by fibroblasts are believed to be the motive forces responsible for generating tension in the collagen fiber network, keeping the gingiva tightly bound to the teeth and the teeth firmly bound to each other and to the alveolar bone. The cytoplasmic apparatus involved in generating tractional forces in connective tissue is described in chapter 6.

Gingival connective tissue contains collagen types I, III, IV, V, VI, and VII. Types I and III form the major collagen fibers, which occupy approximately 60% of the extracellular space.^{1,3,67} Type III fibers are abundant beneath the epithelium and around blood vessels.⁶⁸ Type IV is a component of the basal lamina of gingival epithelium, blood vessels, and neural tissue. Types V and VI are minor components found in association with blood vessels, nerves, and subepithelial basement membranes. Collagen type VI has also been shown to form unbanded microfilaments that bridge larger type I fibrils. Type VII forms the anchoring fibrils, which are part of the subepithelial basement membrane. The self-assembly of types IV and VII collagen is reviewed in chapter 4.

The proteoglycans of gingival connective tissue are similar to those that have been isolated from dermal connective tissue. Decorin, biglycan, and versican have all been identified in gingiva.⁶⁹ Extracellular proteoglycans have an organizing role in the forma-

Fig 5-9 Electron micrograph of lamina propria fibroblast. Note the abundance of rough endoplasmic reticulum (RER) and well-developed Golgi complex (GC). (Col) Collagen; (M) mitochondria; (MP) macrophage; (N) nucleus. (Original magnification $\times 5,400$.)



tion of collagen fibrils. Dermatan sulfate and chondroitin sulfate side chains appear to interconnect collagen fibrils of the gingiva. Dermatan sulfate (decorin) has been localized at the D-band of collagen fibrils in the supra-alveolar fiber apparatus.

Gingival fibroblasts show considerable variation in morphologic development, from highly robust cells containing an abundance of rough endoplasmic reticulum, well-developed Golgi complexes, and mitochondria (Fig 5-9) to fibroblasts that show signs of swelling and degeneration. Such changes probably reflect site-to-site variations in cytokines and other biologic mediators of inflammation. Within inflamed gingival connective tissue, plasma cells are found in clusters and/or in close contact with fibroblasts.⁷⁰ Evidence of fibroblastic lysis was observed in such sites.

During the past decade, evidence that functional heterogeneity exists among phenotypically stable fibroblast populations has been obtained. Although fibroblasts may have the same general shape, abundant rough endoplasmic reticulum, well-developed Golgi complexes, and the same overall cytoskeletal organization, they present functionally distinct subgroups.^{71,72} When grown in cell culture, gingival fibroblasts give rise to subpopulations that respond differently to various stimuli. For example, fibroblasts isolated from free marginal gingiva release greater amounts of glycosaminoglycans and collagen in response to diphenylhydantoin compounds than do their counterparts isolated from attached gingiva (see "Periodontal pocket formation," later in

this chapter). Gingival fibroblasts from the tips of connective tissue papillae retain a fetal migratory phenotype, producing migration stimulatory factor. Skin fibroblasts and fibroblasts from the deeper gingival connective tissue do not produce migration stimulatory factor. The presence of fibroblasts with a fetal migratory phenotype in gingiva may in part explain its rapid wound-healing ability. The significance of diverse fibroblast types in normal connective tissue function and disease remains to be fully explored.⁷³

Gingival fibroblasts perform several functions beyond matrix deposition. They secrete collagenase and are active in matrix degradation.⁷⁴⁻⁷⁶ In vitro studies show that normal human gingival fibroblasts express mRNA for IL-1 β , IL-6, and IL-8⁷⁷ and respond to bacterial lipopolysaccharide by synthesizing IL-1 β , IL-6, and IL-8.^{78,79} By joining with other cells in the elaboration of these proinflammatory interleukins, gingival fibroblasts can function as accessory cells in promoting inflammation and the immune response (see "Primary proinflammatory cytokines and chemokines"). There is also evidence that gingival fibroblasts may switch from a formative to a resorptive phenotype in response to inflammatory cytokines.⁸⁰

Gingival connective tissue fibroblasts develop from perifollicular mesenchyme, a derivative of the stomodeal mesoderm. In contrast, the fibroblasts of the periodontal ligament originate from the dental follicle (sac), a derivative of the neural crest ectomesenchyme. During normal development of the periodontium, gingival fibroblasts do not come into

contact with the tooth surface. In contrast, the fibroblasts of the periodontal ligament become juxtaposed to the tooth surface soon after the disruption of the root sheath. They have the capacity to fabricate an attachment matrix (acellular cementum).

In periodontal surgical regeneration and reattachment procedures, it is recommended that barriers be inserted to exclude gingival fibroblasts (as well as epithelial cells) from gaining access to the root surface and simultaneously create favorable conditions for the repopulation of the root surface by periodontal fibroblasts (see chapter 6). Although gingival fibroblasts have receptors that permit them to bind to the molecular components of the root surface, they appear to be unable to regenerate the periodontal ligament attachment.

In general, gingival connective tissues have a high potential for regeneration. The collagen of gingival connective tissue turns over more rapidly than that of skin and bone but more slowly than that of the periodontal ligament.⁸¹ New fibroblasts are derived from the proliferation of undifferentiated perivascular cells⁸² as well as by division of differentiated fibroblasts.^{83,84}

Supply of Blood to the Gingiva

The gingiva receives its blood supply from three main sources (Fig 5-10):

1. Blood vessels traverse the interdental bony septa and reach the gingiva via foramina in the cortical plate.
2. Other blood vessels reach the gingiva from the periodontal ligament.
3. Supraperiosteal blood vessels of the alveolar mucosa and palate also supply the gingival tissues.^{1,85,86}

Terminal vessels beneath the OGE (and OSE) form a vascular plexus, characterized by hairpinlike terminal capillary loops extending into the dermal papillae between the rete pegs (Fig 5-11).^{85,86} Each loop has an ascending arterial segment and a venous descending segment. The vascular plexus beneath the JE (the gingival plexus) forms a basketlike system of anastomosing terminal vessels (see Fig 5-11). A large portion of this plexus is made up of post-capillary venules. The vessels of the JE gingival plexus are continuous with the vascular bed of the periodontal ligament. The architecture of the terminal vascular bed of the two regions (OGE versus JE) differs in conformity with contours of the two epithelial-mesenchymal interfaces.¹

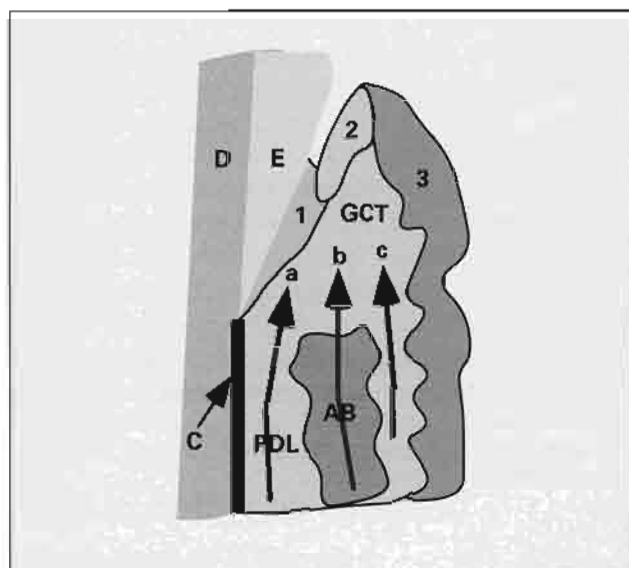


Fig 5-10 Blood supply to the gingival tissues, originating from vessels entering from (a) the periodontal ligament (PDL), (b) the interdental septa, and (c) the gingival connective tissue (GCT). (AB) Alveolar bone; (C) cementum; (D) dentin; (E) enamel; (1) junctional epithelium; (2) sulcular epithelium; (3) oral gingival epithelium.

The endothelial cells of the capillaries and post-capillary venules beneath the JE resemble those that make up high endothelial venules of lymph nodes.^{87,88} The endothelial cells are low cuboidal and have highly developed Golgi complexes and moderate amounts of rough endoplasmic reticulum.^{36,88}

The vascular bed of the gingiva changes in response to inflammation.⁸⁸⁻⁹⁰ Proliferation of endothelial cells increases along with infiltration of lymphocytes in the gingival epithelial-connective tissue border.⁹¹ In acute and chronic gingivitis, the terminal vessels proliferate and dilate, forming tortuous, glomerular-like segments beneath the epithelium. Increased fragility and permeability accompany changes in the shape of the terminal vessels.

A wide variety of exogenous and endogenous factors can stimulate vasodilation of gingival blood vessels. One factor, a cysteine proteinase from *Porphyromonas gingivalis*, generates bradykinin from kallikrein.⁹²

As the permeability of the blood vessels beneath the JE increases during gingival inflammation, the flow of gingival crevicular fluid rises and various plasma proteins appear in the fluid. Bleeding from gingival blood vessels is a sign of advanced gingival

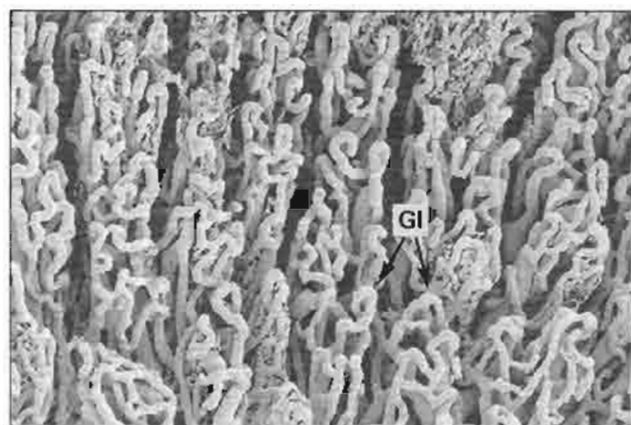


Fig 5-11a Vascular network located beneath the sulcular epithelium. The network consists of glomerular loops (GI) projecting into the ridgelike connective tissue papillae. (Reprinted from Matsuo and Takahashi⁸⁶ with permission. Original magnification $\times 110$.)

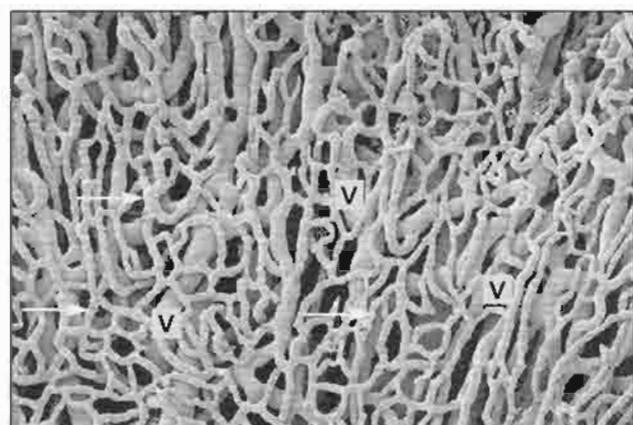


Fig 5-11b Vascular network located beneath the junctional epithelium. The network is in the form of a flat mesh. (arrows) arterioles; (V) venules. (Reprinted from Matsuo and Takahashi⁸⁶ with permission. Original magnification $\times 110$.)

disease, but it is not always a reliable indicator of active periodontal ligament tissue destruction.

Gingival blood vessels undergo vasoconstriction in response to sympathetic nerve stimulation. Blocking receptors for 5-hydroxyhistamine and α_1 -adrenergic receptors on gingival blood vessels can inhibit the vasoconstriction. Severe stress may produce a prolonged decrease in blood flow to gingival tissues, thereby decreasing natural defense systems, which may lead to necrotizing lesions of the marginal gingiva.

Experimentally induced gingival inflammation stimulates endothelial cell proliferation and an increase in numbers of blood vessels.⁹¹ During inflammation, the postcapillary venules of the gingival plexus resemble high endothelial venules. The hypertrophied endothelial cells contain increased amounts of rough endoplasmic reticulum and Golgi membranes. It has been suggested that they may be a significant source of proinflammatory cytokines in gingivitis and periodontitis.⁸⁸ These cells are activated to express increased amounts of surface adhesion molecules, such as ICAM-1, ELAM-1, and leukocyte function antigen 3, which promote the transmigration of leukocytes from the blood to the local connective tissue (see chapters 13 and 14). The presence of IL-1 and LPS during gingival inflammation increases endothelial expression of ELAM-1 and ICAM-1.

Innervation of the Gingiva

Unmyelinated intraepithelial nerves have been localized in the JE, between the basal lamina and the plasma membrane of the basal cells and in the intercellular spaces of basal and midlevel cells (Fig 5-12).^{93,94} The highest concentrations of free nerve endings are in the apical portion of the JE and in the col area.⁹⁵⁻⁹⁷ In comparison, the OSE and OGE are only sparsely innervated. Many nerve bundles and free nerve endings are also located within subepithelial connective tissue.

Most of these nerve endings stem from sensory afferent C- and A-delta fibers associated with nociception. Individual nerve endings are devoid of any Schwann cell covering and contain many small, clear vesicles and larger, dense-core peptidergic granules.⁹⁷ The low-affinity nerve growth factor receptor (p75-NGFR) has been localized in the intraepithelial nerve endings.

Immunocytochemical studies have shown that many of the nerve endings contain substance P and calcitonin gene-related peptide.^{96,98} Substance P acts as a vasodilator, a mitogen for keratinocytes, and an enhancer of phagocytosis by PMNs. Calcitonin gene-related peptide also is a vasodilator. The identification of neuropeptides in gingival free nerve endings opens the possibility that, in addition to transmitting pain sensation, intraepithelial nerve end-

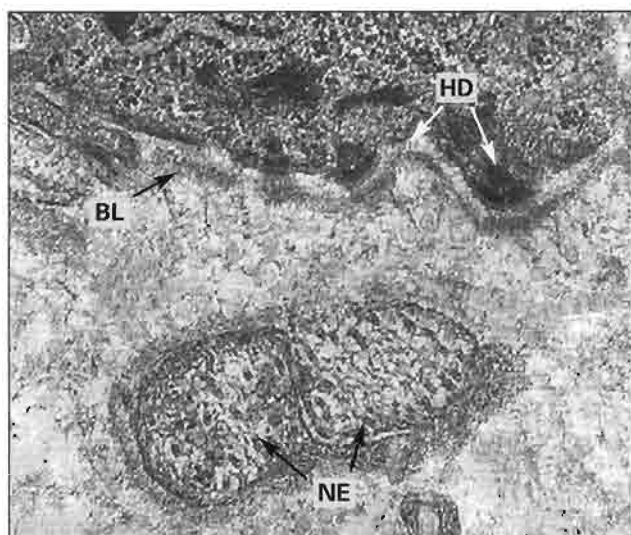


Fig 5-12a Nerve endings (NE) in the lamina propria beneath the basal lamina (BL) of the junctional epithelium. (HD) Hemidesmosomes. (Original magnification $\times 23,000$.)

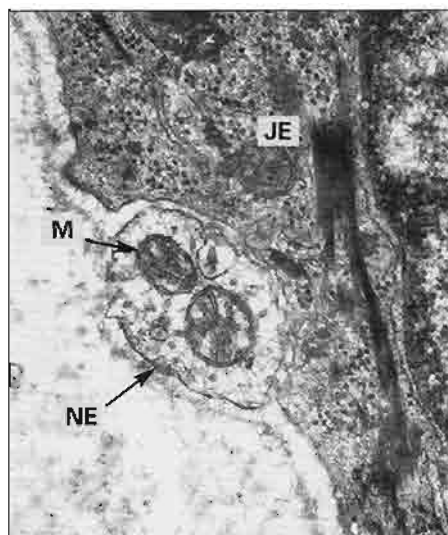


Fig 5-12b Nerve endings (NE) within the epithelial compartment. (M) Mitochondria; (JE) junctional epithelium. (Original magnification $\times 21,000$.)

ings might have a role in modifying cellular activity in the JE as well as in self-modulating receptor sensitivity through the release of specific neuropeptides. It has also been suggested that substance P and calcitonin gene-related peptide, released from sensory nerve endings in the gingiva, stimulate plasma extravasation and the flow of GCF because of their vasodilator effect.⁹⁶

It has been suggested that substance P may have a mitogenic effect on epithelial tissue proliferation during tooth eruption. Nerve endings rich in substance P are found between the reduced enamel epithelium covering the crown of the erupting tooth and the overlying oral epithelium.

Merkel cell neurite complexes (slowly adapting mechanoreceptors) and encapsulated nerve endings (rapidly adapting mechanoreceptors) are also found in the OGE and its lamina propria (see chapter 10).⁹⁹

Flow and Composition of Gingival Crevicular Fluid

Fluid that originates in gingival blood vessels flows through the junctional epithelium into the gingival crevice.^{46,100} In healthy gingiva, the flow of GCF is minimal to absent, but, during inflammation, the rate of flow increases.¹⁰¹ The rate of its flow is correlated to the permeability of gingival blood vessels. During inflammation, blood vessels dilate and plasma enters the connective tissue spaces in greater amounts.

The composition of GCF reflects its origin from plasma and extracellular fluid. Molecular components and metabolites of plaque bacteria contribute to the composition of GCF in the gingival sulcus. Bacterial products, such as endotoxin, can diffuse down their concentration gradients through the JE against the flow of GCF.¹⁰² The many components of GCF include serum proteins, components of the complement system, collagenase, elastase, cathepsins, connective tissue matrix breakdown products, antibodies directed to plaque bacteria, cellular adhesion molecules, interleukins, and prostaglandins.¹⁰³⁻¹¹⁴

Because the flow of GCF and several of its components is increased during periods of inflammation, there have been numerous attempts to use measurements of GCF and its contents as an index of disease severity and activity. Additional research in this area should lead to practical and objective clinical tests for assessing the health of the gingiva and periodontium.

Basic Science Correlations

Cell-to-cell communication by chemical messengers

Multicellular organisms use a wide variety of chemical signals to control and coordinate cellular activities for normal, life-sustaining operations as well as for mounting defenses against foreign organisms. Sig-

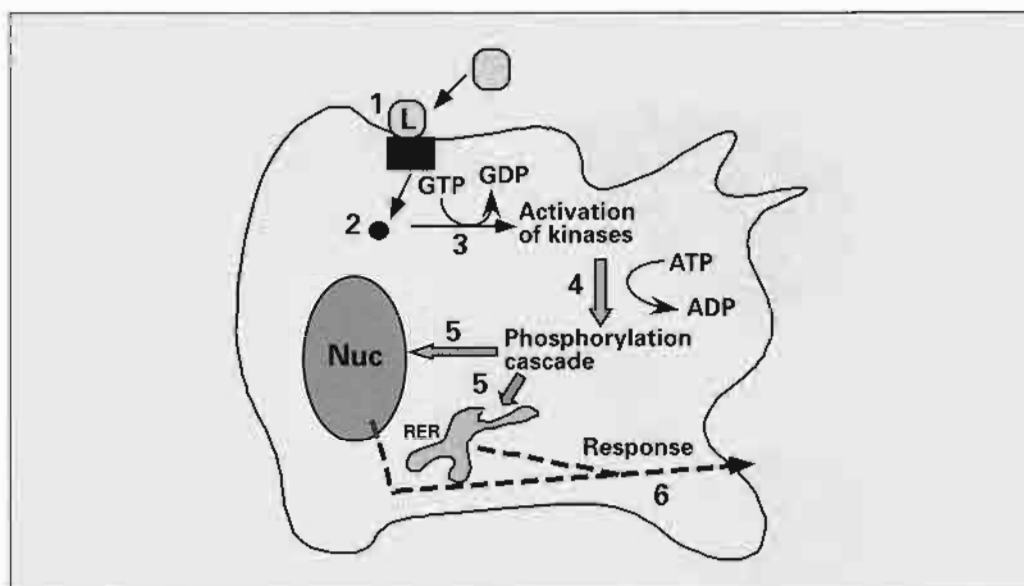


Fig 5-13 Steps in a typical signal transduction event. (1) The signal transduction pathway is activated by interaction of a signaling molecule (L) with its receptor (R). (2) The activated receptor, in association with other plasma membrane constituents or cytoplasmic proteins, generates second messenger molecules that amplify the initial R-L interaction. (3) The second messengers participate in the activation of protein phosphokinases. (4) Kinase activation starts a cascade of phosphorylations of serine- and threonine-containing proteins. (5) These reactions ultimately produce a regulatory effect at the nuclear level or at the level of a cytoplasmic organelle to produce a cellular response, which often involves the secretion of a product (6) that may have an autocrine or paracrine action of its own. The various enzymatic steps are powered by the hydrolysis of guanosine triphosphate (GTP) and adenosine triphosphate (ATP). (ADP) Adenosine diphosphate; (GDP) guanosine diphosphate; (Nuc) nucleus; (RER) rough endoplasmic reticulum.

naling systems require a signal molecule and a corresponding receptor. Most chemical signaling molecules are small, water-soluble peptides that are released into the extracellular environment of a cell. These substances have short half-lives and a limited range of diffusion within the extracellular fluids. Some signaling molecules are hydrophobic substances that diffuse through the lipid bilayers of the plasma membrane to interact with intracellular receptors.

The signal molecule communicates with the receiving cell by binding to a receptor located in the plasma membrane (Fig 5-13). Each receptor protein exhibits an appropriately shaped and electrostatically charged receptor site for a specific signaling molecule (the ligand). The primary receptor-ligand binding reaction, occurring either at the cell surface or within the cytoplasm, generates second messengers that amplify the primary signal.

These signals activate a cascade of enzymatic reactions involving the phosphorylation of cytoplasmic proteins that regulate cytoplasmic and/or nuclear events in the receptor cell (see Fig 5-13). Many signaling molecules (mitogens) increase cell proliferation.

Other signals create a heightened state of synthetic and secretory activity in the responding cells. In either case, the responding cells become "activated." Inhibitory signals downregulate synthetic pathways and/or turn off gene transcription. Each cell type uses hundreds of different receptors and numerous signal transduction pathways to read and respond to its immediate molecular environment.

In its most common form, chemical signaling involves the release of a signaling molecule into the surrounding extracellular fluid and its binding by a receptor on the surface of a neighboring cell (Fig 5-14). This is called *paracrine signaling*. In some cases, the signaling molecule can bind to receptors on the surface of the cell from which it originated; this is called *autocrine signaling* (see Fig 5-14). Paracrine and autocrine signaling molecules are collectively referred to as *cytokines*. *Endocrines* are signaling molecules that travel via the bloodstream from their cell of origin to a wide variety of target cells distributed throughout the body. *Neurotransmitters* are chemical signaling molecules that travel within the cytoplasm of an axon to be released at synaptic junctions

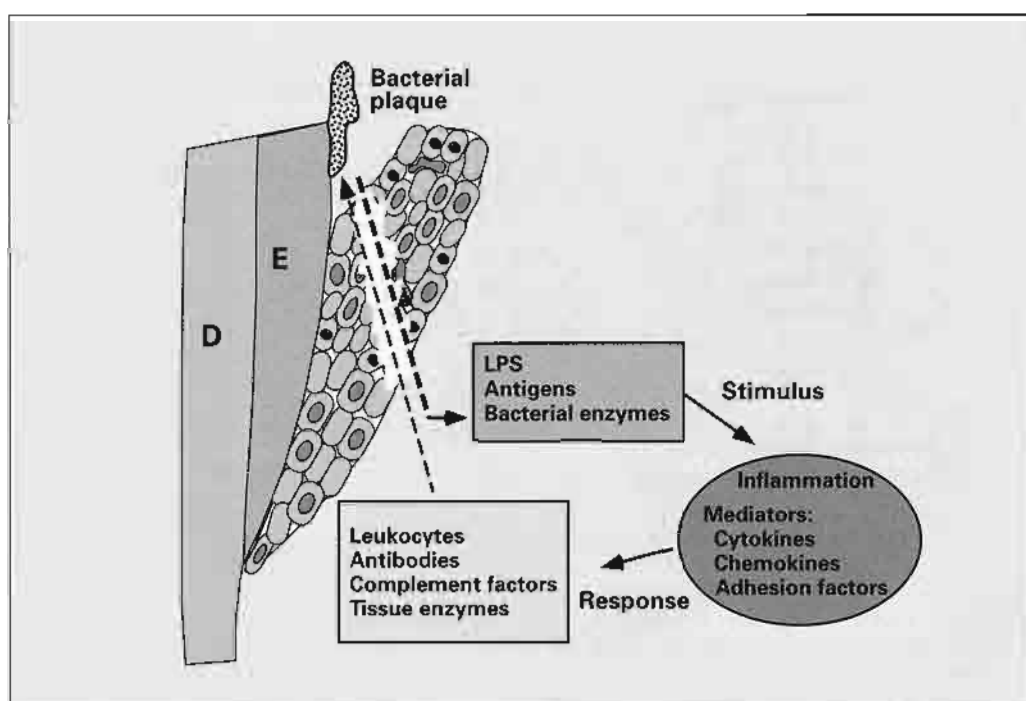


Fig 5-15 Interactions between oral microbiota and gingival tissues. Bacterial antigens, proteolytic enzymes, lipopolysaccharides (LPS), and other water-soluble molecules penetrate the junctional epithelium and enter the connective tissues. These substances activate host cells to produce mediators of inflammation, which coordinate a response involving the local differentiation of T and B cells, increased leukocyte transmigration and phagocytosis, and the activation of extracellular enzyme systems. (D) Dentin; (E) enamel.

cally active molecules, such as prostaglandin E_2 , $TNF-\alpha$, reactive oxygen metabolites, and interferon γ .¹²⁵⁻¹²⁷ The positive correlation between increased gingival inflammation and the growth of Gram-negative bacteria in the gingival sulcus underscores the potential pathogenic significance of the LPS-IL-1 response.¹²⁸ Exotoxins secreted by Gram-positive microorganisms also stimulate the expression of IL-1. Macrophages, fibroblasts, and gingival epithelial cells expressing IL-1 and IL-6 have been localized in inflamed human gingiva.¹²⁹⁻¹³¹

The importance of IL-1 as a key regulator of gingival and periodontal inflammation has been underscored by numerous investigators.^{119,132} A specific allele (allele 2) of the IL-1 β gene appears to be associated with advanced periodontitis.¹³³ Furthermore, the ratio of IL-1 to IL-1-receptor antagonist in gingival tissue is increased in sites affected by periodontal disease.

Cell surface receptors for IL-1 are found on keratinocytes, fibroblasts, endothelial cells, and most members of the leukocyte family.¹²⁹ These cells appear to be extremely sensitive to activation by IL-1, because only 2% of the cell's IL-1 receptors have to be occupied to obtain a response. A variety of signal

transduction pathways are activated by IL-1 and its receptor to elicit fast responses, such as the production of prostaglandin E_2 , and slower responses, involving protein phosphorylations and downstream gene transcription.

Interleukin 1 inhibits the expression of procollagen mRNA and stimulates gingival fibroblasts to secrete collagenase and prostaglandins.¹³⁴⁻¹³⁷ It also increases the amount of ICAM-1 on gingival fibroblasts which may be important in retaining various inflammatory cells within the local connective tissues.^{138,139} Furthermore, osteoclastic bone resorption is increased by IL-1 β .^{120,140} Interleukin 1 β has a direct stimulatory effect on osteoclasts as well as an indirect effect through its ability to increase the expression of IL-6 by osteoblasts. Cells expressing IL-6 are present in the connective tissue adjacent to active periodontal bony lesions. Interleukin 1 increases the expression of ELAM-1 and ICAM-1, key molecules for neutrophil margination and transmigration.

Key gene transcription products induced by IL-1 include two chemoattractant cytokines (chemokines), interleukin 8 (IL-8) and monocyte chemoattractant protein 1 (MCP-1).^{124,141} Recent studies suggest that

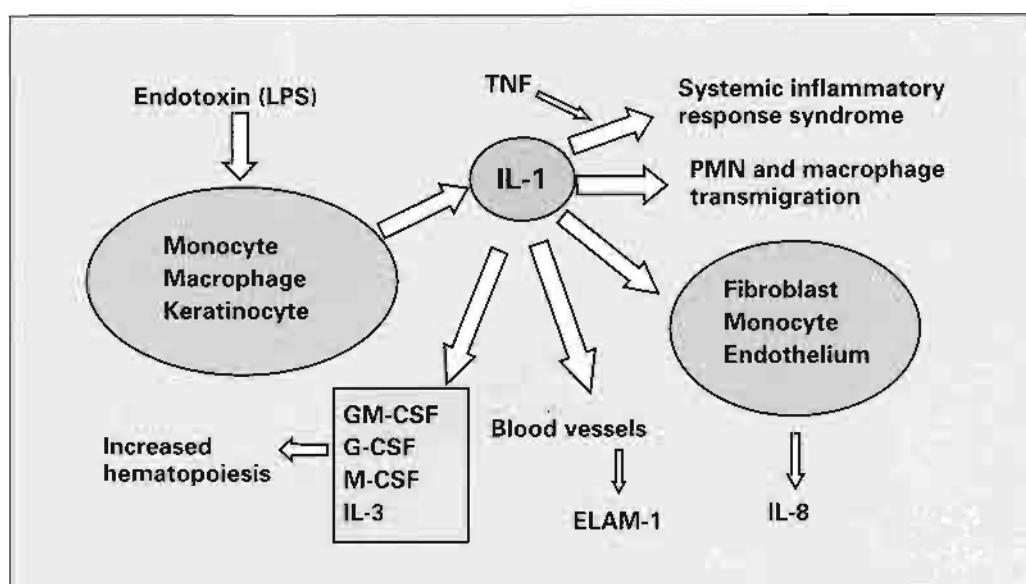


Fig 5-16 Action of interleukin 1 (IL-1), a key regulator of the inflammatory response. Tumor necrosis factor (TNF), another member of the cytokine family, shares many of the properties of IL-1. At the systemic level, IL-1 and TNF produce an inflammatory response syndrome with fever, hypotension, and vasculitis. Local effects of IL-1 that are significant to gingival inflammation include elevated neutrophil chemotaxis, increased expression of endothelial adhesion molecules, and the production of IL-8 and monocyte chemoattractant protein by several cell types. IL-1 also plays a role in stimulating hematopoiesis through increased production of various colony-stimulating factors. (LPS) Lipopolysaccharide; (ELAM-1) endothelial leukocyte adhesion molecule 1; (PMN) polymorphonuclear leukocyte; (GM-CSF) granulocyte-macrophage colony-stimulating factor; (G-CSF) granulocyte colony-stimulating factor; (M-CSF) macrophage colony-stimulating factor.

the activation of chemokine expression by IL-1 in fibroblasts, oral keratinocytes, macrophages, and endothelial cells may play a significant role in gingival inflammation.^{124,142}

Chemokines have the following characteristics:

1. They are secretory, low-molecular weight proteins.
2. They are secondary proinflammatory mediators.
3. They have a chemoattractant effect on polymorphonuclear neutrophils.

There are two subfamilies of chemokines, based on the position of cysteine residues in the polypeptide chain. The CXC (α) chemokines, including IL-8, have an intervening amino acid located between the first two cysteine residues. Interleukin 8 and other CXC chemokines attract and stimulate neutrophils. In the CC (β) chemokines, the first two cysteines are positioned adjacent to each other. Monocyte chemoattractant protein and other members of the CC subfamily attract and stimulate monocytes and lymphocytes.¹⁴³

Monocyte chemoattractant protein 1 is believed to be partly responsible for the large monocyte-

macrophage infiltration of gingival connective tissue in early gingival inflammation. Oral keratinocytes produce IL-8 when stimulated by TNF- α or interferon γ .¹⁴⁴ Endothelial cells and fibroblasts express IL-8 and MCP-1 when exposed to LPS (Fig 5-17). Peripheral blood mononuclear phagocytic cells express IL-8 and MCP-1 when exposed to a wide variety of oral microbial products. Both IL-8 and MCP-1 have been localized in inflamed gingival tissue and GCF.¹⁴⁵ Plaque bacteria have been shown to stimulate the secretion of IL-8 by gingival fibroblasts and macrophages.

Interleukin 8 is capable of effecting a wide spectrum of biologic responses, including the migration and phagocytic activity of neutrophils. In vitro studies suggest that the transepithelial migration of neutrophils is regulated by the capacity of the epithelial cells to produce IL-8.¹⁴⁶ Fibroblasts lose focal adhesions and assume a migratory phenotype when exposed to IL-8 in vitro.¹⁴⁷ The administration of IL-8 to host tissues leads to numerous inflammatory changes, such as plasma leakage and edema, characteristics of gingival inflammation.

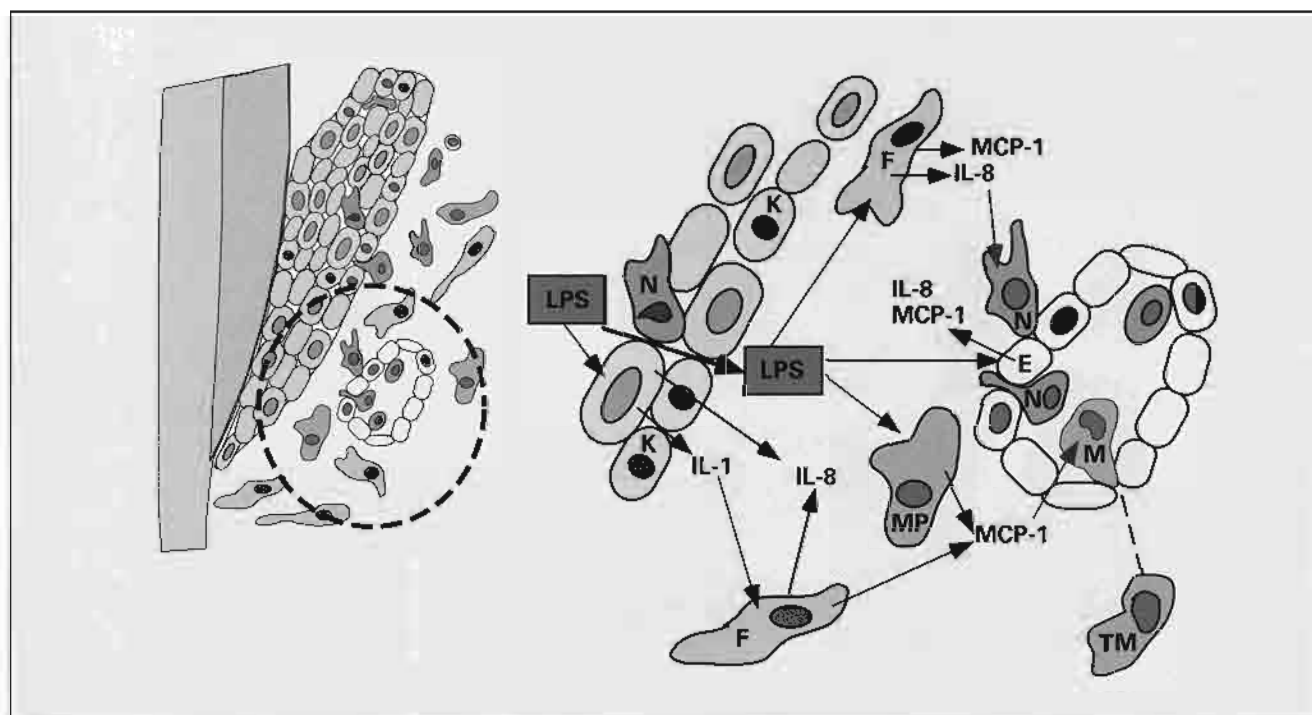


Fig 5-17 Importance of bacterial lipopolysaccharide (LPS) in generating the production of interleukin-1 (IL-1) by keratinocytes (K). The secondary proinflammatory chemokines, IL-8 and monocyte chemoattractant protein (MCP-1), are expressed by fibroblasts (F), endothelial cells (E), and macrophages (MP) in response to activation by contact with LPS or IL-1. Neutrophil (N) transmigration out of local blood vessels is increased in response to IL-8. Monocyte chemoattractant protein has a chemotactic and activating effect on monocytes (M). The histopathology of the early gingivitis lesion, characterized by steep increases in the number of neutrophils and macrophages, underlines the significance of proinflammatory cytokines and chemokines in regulating the host response to oral bacteria and their products. (TM) Tissue monocyte.

Clinical Correlations

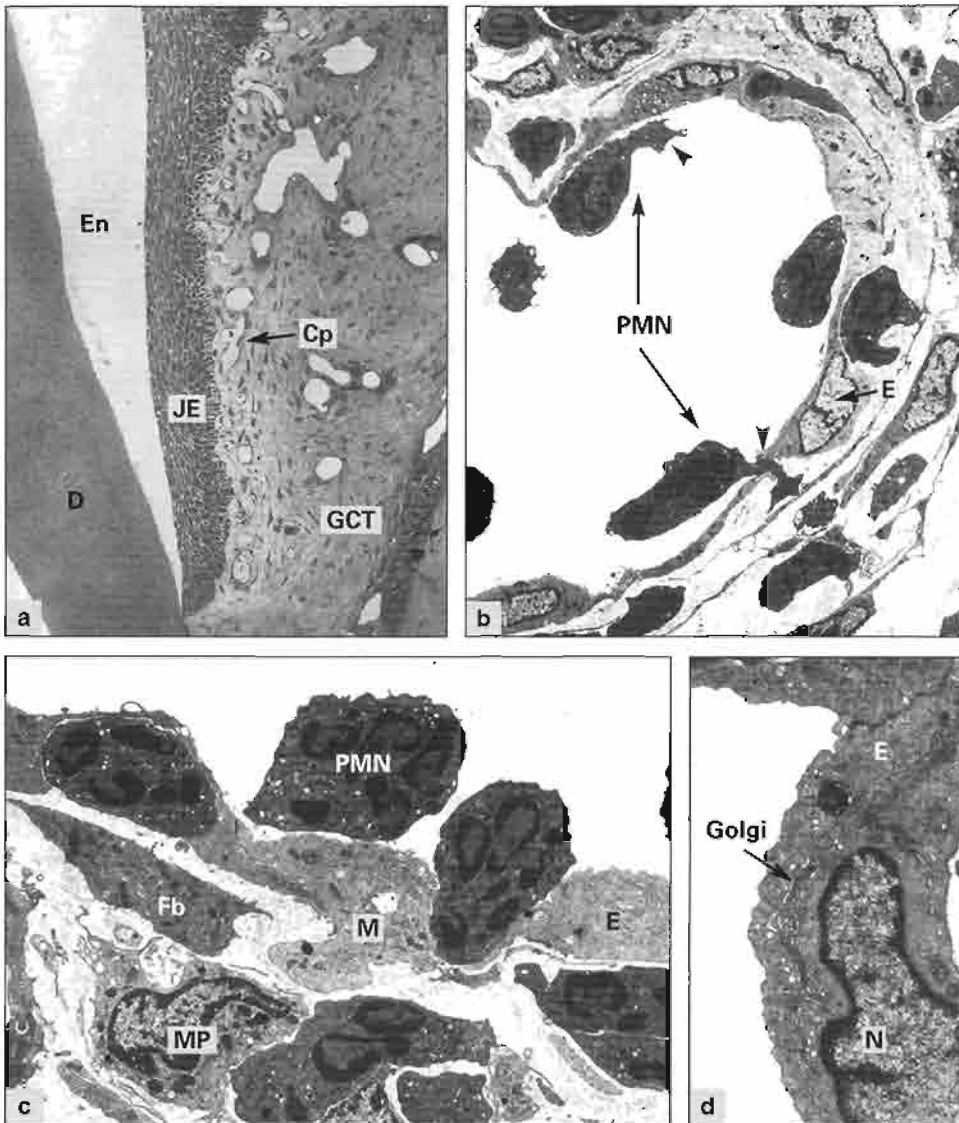
Inflammation and tissue destruction

A wide range of cellular and humoral mechanisms protect the body from foreign organisms, whether they be microorganisms, viruses, or cancer cells. These defensive mechanisms can be activated solely by the intrusion of molecules produced by the foreign organisms, without the actual penetration of the offending parent organisms into host tissues (see Fig 5-15). Defensive responses are regulated by a wide variety of chemical messages, many stemming from circulating cells that are recruited into the area and still others from resident epithelial cells, fibroblasts, and endothelial cells in the area under attack.¹⁴⁸

Tissue destruction is an unwanted by-product of inflammation. Fortunately, in most cases, the offenders are destroyed and damaged tissue is subsequently regenerated. Clinically, this constitutes acute inflammation followed by rapid healing response. On the other hand, there are instances when the body's

defensive mechanisms are unable to overpower the foreign organisms, and there is a long, slow conflict, characterized by chronic inflammation and extensive tissue damage.

Gingivitis and periodontal disease provide excellent examples of chronic inflammation and tissue destruction induced by bacterial and host cell mediators.^{149,150} Gingivitis and periodontitis exist as chronic conditions because the body's defensive systems are unable to reject the offending microorganisms once they have become established in large adherent colonies (plaque). Most oral bacteria live exclusively external to host tissues in a niche, such as the gingival sulcus, which is rich in nutrients. There they can multiply rapidly and withstand the efforts of neutrophilic leukocytes, immunoglobulins, and other defensive molecular strategies of the host. Although individual bacteria at the surface of the plaque are phagocytosed and killed by neutrophils, massive accumulation of bacteria will occur in time in most individuals, unless good oral hygiene is practiced.



Figs 5-18a to 5-18d (a) Junctional epithelium (JE) with its gingival connective tissue (GCT) and capillary plexus (Cp). (D) Dentin; (En) demineralized enamel space. (Original magnification $\times 120$.) (b) Portion of a postcapillary venule of the lamina propria depicting margination and transmigration of neutrophils (PMN). Note the leading pseudopod (arrowheads) of the homing neutrophils. (E) Endothelial cell. (Original magnification $\times 1,600$.) (c) Cluster of PMNs and a monocyte-like cell (M) in various phases of endothelial penetration. (Fb) Fibroblast; (MP) macrophage; (E) endothelial cell. (Original magnification $\times 3,400$.) (d) High magnification of the well-developed Golgi complex of the endothelial cells (E) of the gingival lamina propria. (N) Nucleus. (Original magnification $\times 11,300$.)

It is estimated that approximately 300 species of bacteria are indigenous to the oral cavity. Many of these species survive and flourish because of their ability to secrete extracellular matrices that cause them to adhere to solid substrates, such as the surfaces of epithelial cells, enamel, or cementum. Other species establish themselves by association with the already adherent pioneer organisms. Through this process, microbial plaque develops along tooth surfaces, especially around the cervical margin.

Microbial products gain access to underlying host tissues via the gingival sulcus and JE. Following penetration of the JE, these products stimulate the cellular and humoral immune systems of the host. A prominent aspect of the innate or cellular response is the constant transmigration of neutrophils from the blood vessels of the JE plexus and the movement of these neutrophils through the JE to the sulcus (Fig 5-18). In a healthy individual practicing a reasonably good level of oral hygiene, a dynamic equilibrium is

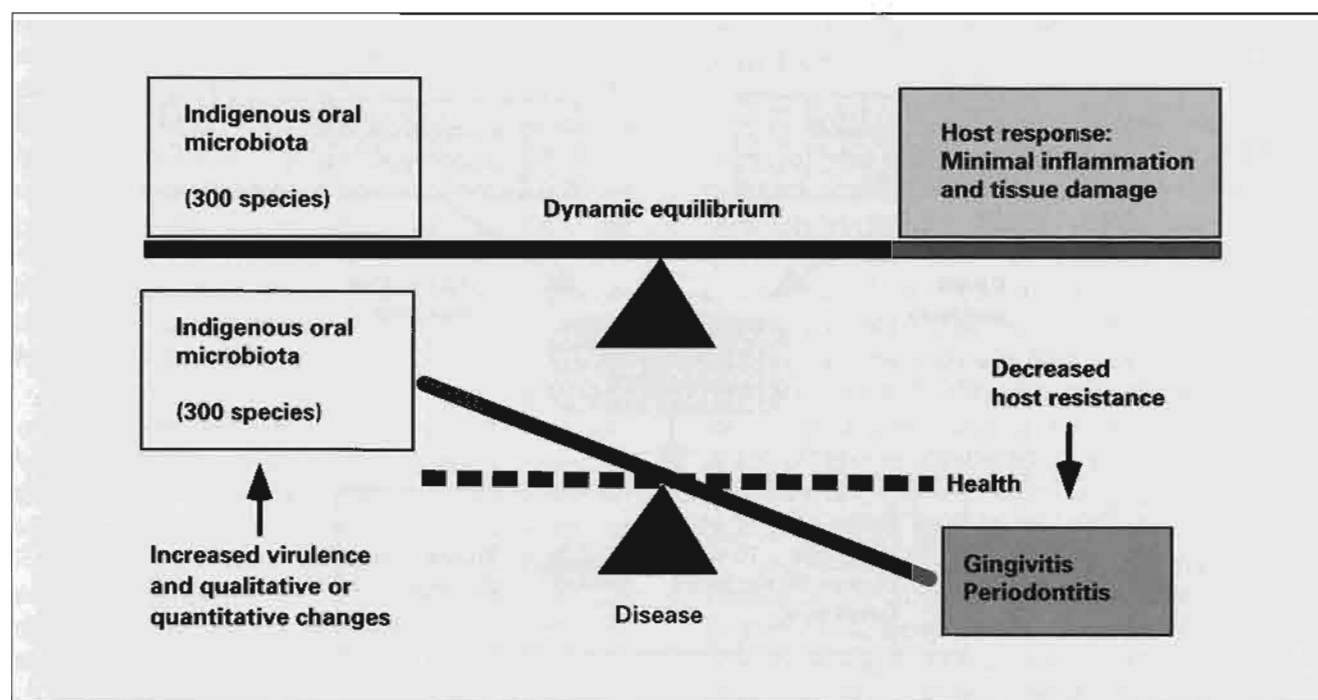


Fig 5-19 Balance between microbial and host factors in the gingival environment.

established, and the presence of these bacteria causes only minimal inflammation and tissue damage (Fig 5-19). This equilibrium can be disturbed by factors such as an alteration of the bacterial flora, arising from virulent transformation of a species; unregulated expansion of a particular subset of organisms; excessive growth of the total number of microorganisms; or a decreased ability of the host to mount an effective defense. Destructive inflammation develops whenever the balance is tilted in favor of the microorganisms.

Oral bacterial products that damage the cells and tissues of the host include exotoxins, endotoxins, proteolytic enzymes, ammonia, and polyamines.^{149,151} Lipopolysaccharides and bacterial antigens trigger host cell responses that include increased expression of proinflammatory cytokines, increased proliferation and migration of inflammatory cells, and the secretion and activation of matrix metalloproteinases.^{74,152}

The site of interaction between the microbial product and the host can be within the gingival sulcus, within the junctional and sulcular epithelial lining, or within the extracellular spaces of the gingival connective tissue. The penetration of the JE by lipopolysaccharide (endotoxin), bacterial antigens, and proteolytic enzymes is especially significant in generating

a local inflammatory response (see Fig 5-15).^{153,154} The mechanisms that activate and regulate the inflammatory process are highly complex. There are numerous end products of inflammation that help protect the host against bacteria and their products. However, many of these end products have the potential to cause significant injury to host tissues.¹⁵⁵

The host defenses consist of acquired immune responses (see chapter 13) and innate defensive systems (see chapter 14). The complement system, composed of 20 enzymatic proteins of serum origin, is a formidable component of the innate defense system. When the complement system is activated by antigen-antibody complexes in the classic direct pathway, or by LPS in the alternative pathway, a cascade of proteolytic reactions is triggered to form several biologically active proteins. Many of the reaction products protect the host against bacteria. Complement components coat (opsonize) the surface of microorganisms to make the bacteria more susceptible to phagocytosis. Other complement products form a membrane attack complex that ruptures the outer membranes of Gram-negative bacteria. Small cleavage products (C3a and C5a) produced during complement activation are highly chemotactic for neutrophils. These substances also activate neutrophils

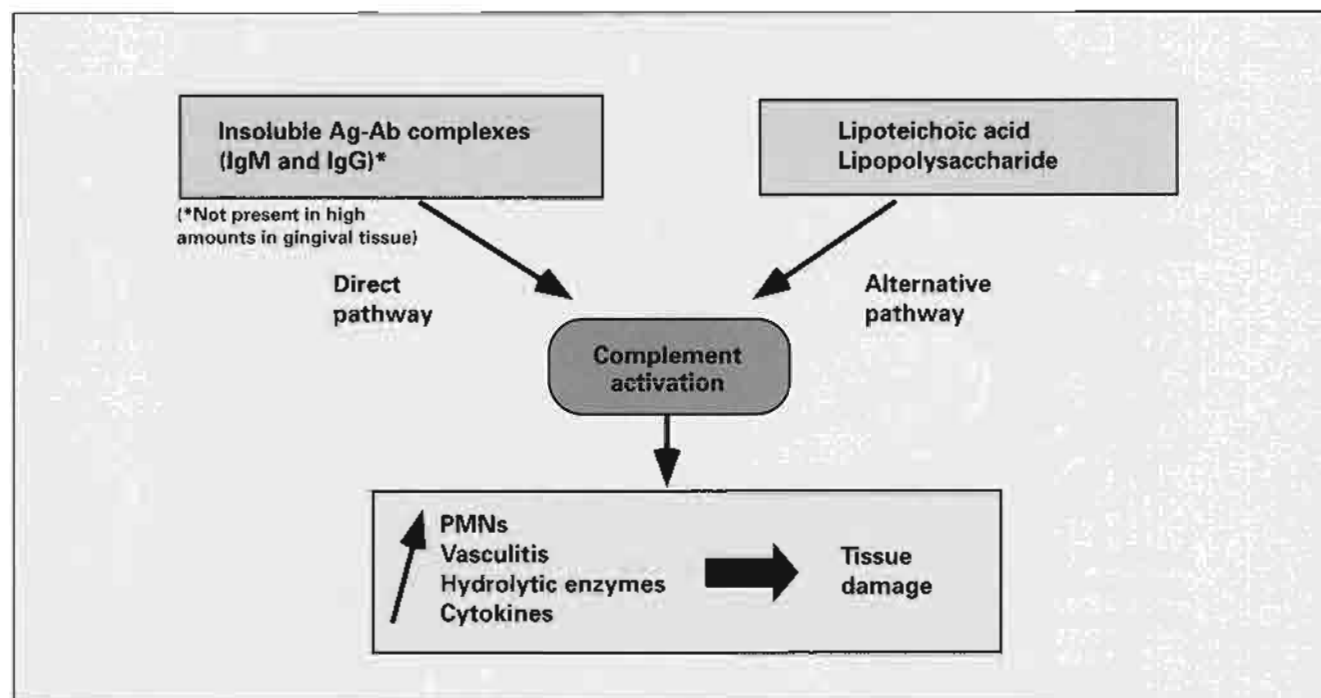


Fig 5-20 Host-mediated tissue injury. Activation of the complement system, either through the direct pathway by antigen-antibody (Ag-Ab) complexes or via the alternative pathway by lipopolysaccharide or lipoteichoic acid, leads to inflammatory changes such as vasculitis, activation and chemotaxis of polymorphonuclear neutrophils (PMNs), and the release of metalloproteinases from neutrophils and macrophages. (IgM) Immunoglobulin M; (IgG) immunoglobulin G.

and macrophages to produce leukotrienes and prostaglandins and to release granules containing proteolytic enzymes.

Unfortunately these substances can cause tissue damage as an unwanted side effect. Neutrophil proteases, released during phagocytosis, can damage vascular basement membranes and the extracellular matrix. For example, elastase, one of many neutrophil proteolytic enzymes, is increased in the tissues and crevicular fluid of patients with gingivitis and periodontitis.¹⁰⁵ Components of the basal lamina and elastic fibers are degraded by elastase. Another consequence of complement activation is local vasculitis, accompanied by neutrophil accumulation and damage to the extracellular matrix (Fig 5-20).

Components of an activated complement system are found in GCF. The spectrum of complement reaction products contained in GCF suggests that the alternative pathway of complement activation predominates in the gingival environment. A more detailed discussion of the complement system is contained in chapter 14.

The generation of plasmin from plasminogen within inflamed gingival tissues is believed to be a significant pathway for the activation of metallopro-

teinases and the subsequent destruction of extracellular matrix.¹⁰³ Macrophages and fibroblasts express the enzyme, plasminogen activator, that converts plasminogen into plasmin. Lipopolysaccharide stimulates the expression of plasminogen activator in human gingival fibroblasts.¹⁵⁶ Secretion and activation of metalloproteinases, and their role in connective tissue destruction, are discussed in chapter 6.

Gingival fibroblasts, as well as macrophages, contain high levels of lysosomal cathepsins, proteolytic enzymes capable of degrading matrix proteins in an acid environment. Studies of inflamed gingival tissues have shown increased numbers of fibroblasts and macrophages rich in cathepsins beneath the junctional and sulcular epithelium. Increased levels of cathepsin D in the GCF of patients with periodontitis were shown to be positively correlated to probing depth and bone loss.¹⁵⁷

Periodontal pocket formation

Gingivitis and periodontitis are characterized by the formation of gingival and periodontal pockets. Pockets are formed from progressive degradation of the connective tissue attachment to the root surface, ac-

accompanied by junctional epithelial cell proliferation and migration along the denuded root surface. This process causes the normally shallow gingival sulcus to deepen, and often to extend apically beyond the crest of the alveolar bone, to create an infrabony pocket. The onset of this disease process and its degree of advance varies from site to site (tooth surfaces). Most periodontal researchers are of the opinion that destruction of periodontal tissue progresses during relatively short periods of activity followed by longer quiescent intervals.^{36,158}

Periodontal disease is typically assessed by measuring probing depth with a thin probe and by visualizing bone loss on dental radiographs. These approaches provide information on past disease but are of limited usefulness for detecting active sites or in predicting future tissue destruction. For this reason, there have been numerous attempts to measure specific components of GCF as indicators of disease activity.

Gingival crevicular fluid is collected from specific sites (for example, the distal gingival sulcus of a molar or a mesiobuccal periodontal pocket), and the test substance is quantified. The concentration of the test substance is then correlated to a measure of tissue destruction. If a positive correlation is found between the level of the test component, for example collagenase, and the measure of ongoing tissue destruction, such as loss of attachment (probing depth), then a potentially reliable test may be developed. Several measurements over time at a specific site are needed to establish the predictive value of the test and to confirm the role of the tested substance as a mechanism of pathogenicity. Studies of this type have demonstrated that there is a high site-to-site variability in the levels of specific enzymes and/or inflammatory mediators, and only a moderate correlation with the severity of tissue destruction. An exception is the correlation of neutrophil collagenase and interleukin-1 β to active tissue destruction. More often than not, however, a positive correlation is only detected when the means of grouped data are compared.

These results underscore the complexity of the inflammatory response and the multifactorial nature of periodontal tissue destruction. Although there is much to be discovered, it is now apparent that knowledge of the interrelationships among the inflammatory cytokines and chemokines, the complement system, and the activation of metalloproteinases is of prime importance to understanding tissue destruction during chronic gingivitis and periodontitis.

Gingival response following tooth eruption

The JE and the subepithelial connective tissue of newly erupted teeth in young children and adolescents are heavily infiltrated with small and medium T lymphocytes. Roughly 75% of all infiltrating cells are lymphocytes, with CD4⁺ outnumbering CD8⁺ cells by a ratio of 2.1:1. Activated macrophages also form a small but significant percentage of the cell infiltrate, while B cells, plasma cells, and mast cells represent a relatively minor fraction. Fibroblasts within the narrow strip of subepithelial connective tissue adjacent to the JE are outnumbered almost 50 to 1 by lymphocytes, and many show evidence of ongoing cell damage.

The presence of this lymphoid infiltrate has been viewed as the earliest stage in the development of gingivitis and periodontitis. However, it has often been observed that there may be little in the way of clinical inflammation associated with this histopathologic picture. Recently, a parallel between this gingival infiltrate and the palatine tonsil has been drawn, and the T-cell infiltrate has been reinterpreted to be a normal defensive response, as opposed to an early pathologic change. Additionally, the gingival connective tissue of germ-free animals contains an infiltrate of lymphocytes, PMNs, and plasma cells.^{38,159} These cells represent a line of defense against the penetration of the JE and OSE by foreign antigens. At what point this defensive infiltrate converts into a tissue-destructive force remains as one of the key questions for periodontal researchers.

Gingival overgrowth

Fibrotic enlargement of the gingiva occurs as a hereditary condition known as *hereditary gingival fibromatosis*.¹⁶⁰ In this condition, gingival fibroblasts express and respond to TGF- β by increasing the secretion of extracellular matrix proteins.¹⁶¹

Gingival overgrowth, characterized by fibroblast proliferation, epithelial hyperplasia, increased deposition of extracellular matrix, and inflammatory cell infiltration, occurs as an unwanted side effect to several therapeutic drugs.¹⁶² Phenytoin, used in the treatment of epilepsy, and cyclosporin A, used as an immunosuppressor in organ transplant procedures, have the potential for causing gingival overgrowth.^{163,164} Phenytoin has been shown to potentiate the action of TNF- α in producing IL-1 β and prostaglandin E by gingival fibroblasts,¹⁶⁵ while cyclosporin A has been shown to elevate platelet-

derived growth factor in gingiva.¹⁶³ Platelet-derived growth factor stimulates proliferation of fibroblasts and the secretion of extracellular matrix. The net effect of the elevated levels of IL-1 β and prostaglandin E appears to be an alteration of the composition of the extracellular matrix and an increase in the inflammatory response.

Calcium antagonists, such as nifedipine, diltiazem, and verapamil, used in the treatment of heart disease, have also been reported to cause gingival overgrowth.¹⁶⁶ Although interference with intracellular calcium-dependent cascades is probably involved, the exact mechanism of action that produces gingival overgrowth is unclear. In vitro studies have shown increased proliferation of fibroblasts, with up-regulation of sulfated glycosaminoglycan, fibronectin, and collagen deposition, in response to some of these drugs.¹⁶⁷ Cyclosporin A stimulates secretion of platelet-derived growth factor β by gingival macrophages, which in turn acts as a promoter of fibroblast proliferation.¹⁶³ Other evidence suggests that these drugs select and amplify fibroblast subpopulations (responders) that are more responsive to local growth factors.

Recent studies indicate a positive correlation between the expression of HLA-DR2 antigens and gingival overgrowth. Patients with the HLA-DR2 genotype had approximately a 15 times greater chance of developing gingival overgrowth than did patients with the HLA-DR1 genotype.

Investigators have also focused attention on the blockage of aldosterone synthesis in the adrenal cortex by calcium channel antagonists.¹⁶⁸ Feedback mechanisms via the pituitary hormone, corticotropin, cause hyperplasia of the adrenal cortex and a subsequent increase in the production of testosterone, a hormone that is capable of stimulating extracellular matrix production by gingival fibroblasts.

Gingival overgrowth leads to formation of pseudo-pockets and accumulation of bacterial plaque. In severe cases, the enlarged gingiva may interfere with normal occlusion. In such cases, excess tissue must be removed surgically and sound oral hygiene must be practiced to minimize the development of inflammation.

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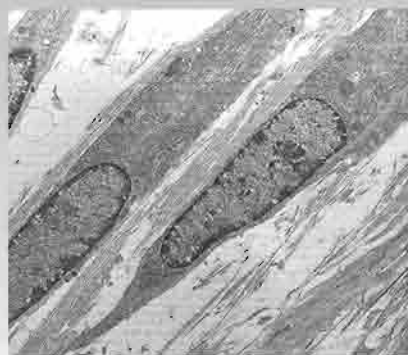
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Chapter 6

Periodontal Ligament



Development and General Structure of the Periodontal Ligament

The periodontal ligament (PDL) is derived from dental sac tissue investing the tooth germ. This tissue, like that of the dental papilla with which it is continuous, is derived from cranial neural crest. Thus, PDL fibroblasts are of ectomesenchymal origin, unlike gingival fibroblasts, which are derived from general mesenchyme. Gingival and PDL fibroblasts demonstrate several different characteristics. Periodontal ligament fibroblasts have a higher rate of proliferation and express greater alkaline phosphatase and cyclic adenosine monophosphate than do gingival fibroblasts.¹

There are at least two lineages of PDL fibroblasts: a common connective tissue fibroblast and an osteoblast-like fibroblast. The latter cell type is considered to give rise to bone cells and cementum. (Several highly recommended reviews of the biology of the PDL have appeared since the publication of Schroeder's monograph.²⁻⁴)

Development of the major collagen bundles, the principal fibers of the PDL, is closely correlated to root formation. Fiber bundles originate at the surface of the newly formed root dentin in close relation to elongated and highly polarized fibroblasts (Fig 6-1).

These nascent fiber bundles (fringe fibers) are tightly packed (bundled) by the action of cementoblasts during the initial development of acellular extrinsic fiber cementum (see chapter 7). Similar fringe fiber bundles originate along the bone surface.

During tooth eruption, as the PDL matures, the fringe fibers merge across the width of the ligament to form the principal fiber bundles (Figs 6-1 and 6-2). In the middle of the PDL, the collagen fiber bundles are less tightly packed. In general, the majority of the principal fibers course in a coronal direction from cementum to bone, forming the oblique fiber group (see Fig 6-2).

During the development of the fringe fibers, fibroblasts exhibit cytoplasmic polarity toward the root and alveolar bone surfaces. The ultrastructural appearance of these cells is consistent with directed cell migration toward each of these surfaces concurrent with the deposition of a collagen- and proteoglycan-rich extracellular matrix (ECM).⁵ A specific cementum attachment protein may favor PDL fibroblast attachment to the cementum surface.⁶

The developing PDL, as well as the mature PDL, contains undifferentiated stem cells that retain the potential to differentiate into osteoblasts, cementoblasts, and fibroblasts.^{7,8} Experimental studies suggest that stem cells occupy perivascular sites in the PDL and in adjacent endosteal spaces.^{8,9} Stem cell progeny undergo further maturation during migra-

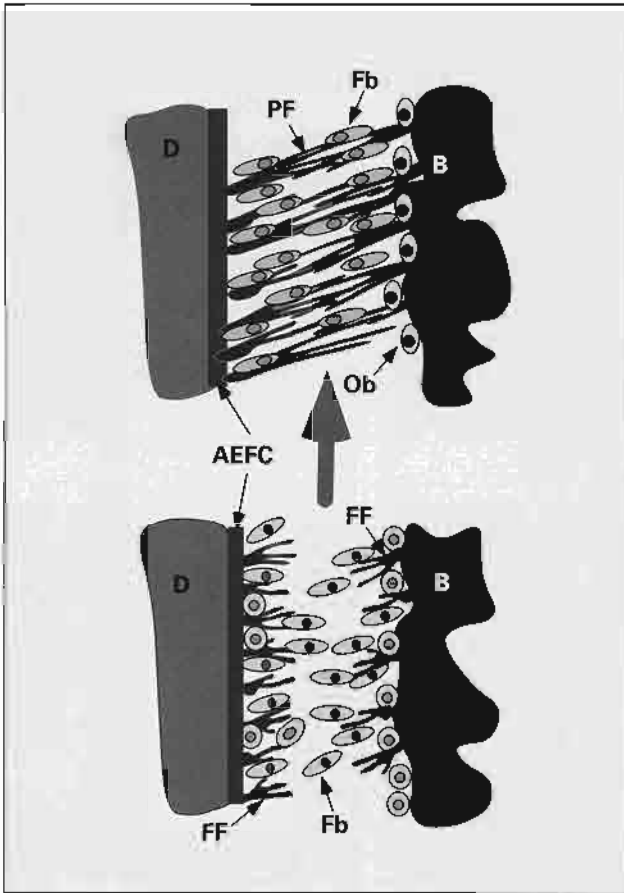


Fig 6-1 Two stages in the development of the principal fibers of the periodontal ligament. Fibers develop as short fringe fibers (FF) extending outward from the surface of bone and acellular extrinsic fiber cementum (AEFC) covering the root dentin (D). As formation nears completion, the splayed ends of the principal fibers (PF) intermingle and merge in the middle of the periodontal ligament. Numerous highly developed fibroblasts (Fb) occupy spaces between the collagen fibers at all stages of formation. (B) Bone; (Ob) osteoblast.

tion to the bone and cemental surfaces.^{10,11} Whether osteoblasts, cementoblasts, and fibroblasts originate from a common ancestor or from a specific line of progenitor cells remains to be clarified.

With continued development of the root, major collagen bundles (the principal fibers) are established as continuous structures embedded as Sharpey's fibers in bone and cementum (Figs 6-1 to 6-3). Sloan and Carter have reviewed the structural organization of the fibers of the ligament.¹² Histologic sections reveal the following distinct groups of principal fibers: dentogingival, alveolar crest, transseptal, interradicular, horizontal, oblique, and apical (see Figs 6-2 and 6-3). The oblique fibers, occupying

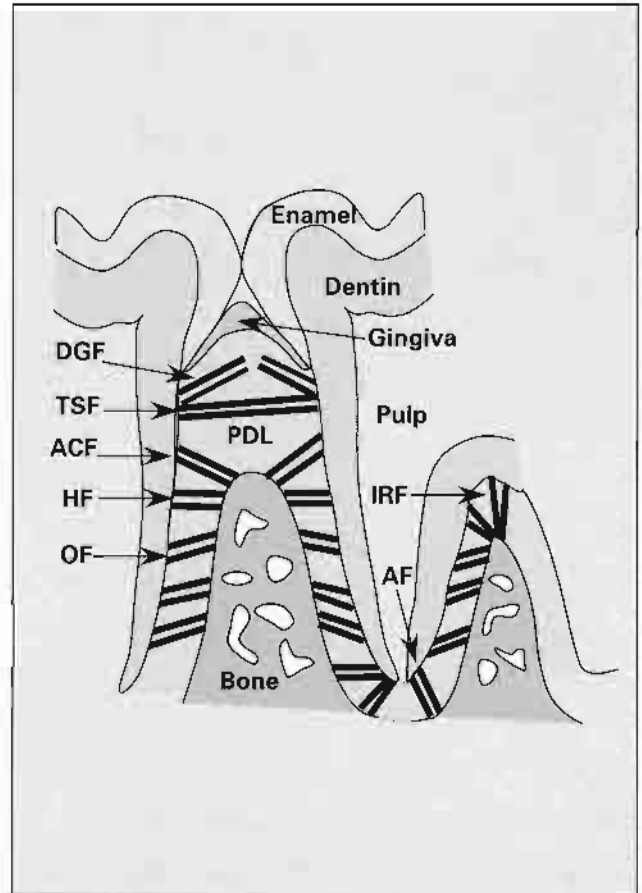


Fig 6-2 Distribution and orientation of the various groups of principal fibers in a mesiodistal view of molar interdental area. (ACF) Alveolar crest fibers; (AF) apical fibers; (DGF) dentogingival fibers; (HF) horizontal fibers; (IRF) interradicular fibers; (OF) oblique fibers; (PDL) periodontal ligament; (TSF) transseptal fibers.

nearly two thirds of the ligament, are inserted in bone coronal to their insertion in cementum. This geometric arrangement of the oblique fibers is ideally suited to absorb intrusive forces generated during mastication.

Biophysical studies of the PDL indicate that it behaves as a viscoelastic system comprising several components. Collagenous fibers, proteoglycans, and tissue fluid combine to cushion the tooth in its alveolus and to resist the forces of mastication. To attach the tooth in its alveolus, the fibers must be embedded in mineralized bone and cementum. A nonfibrillar matrix that stains with ruthenium red appears to "cement" the terminus of the fringe fibers (Sharpey's

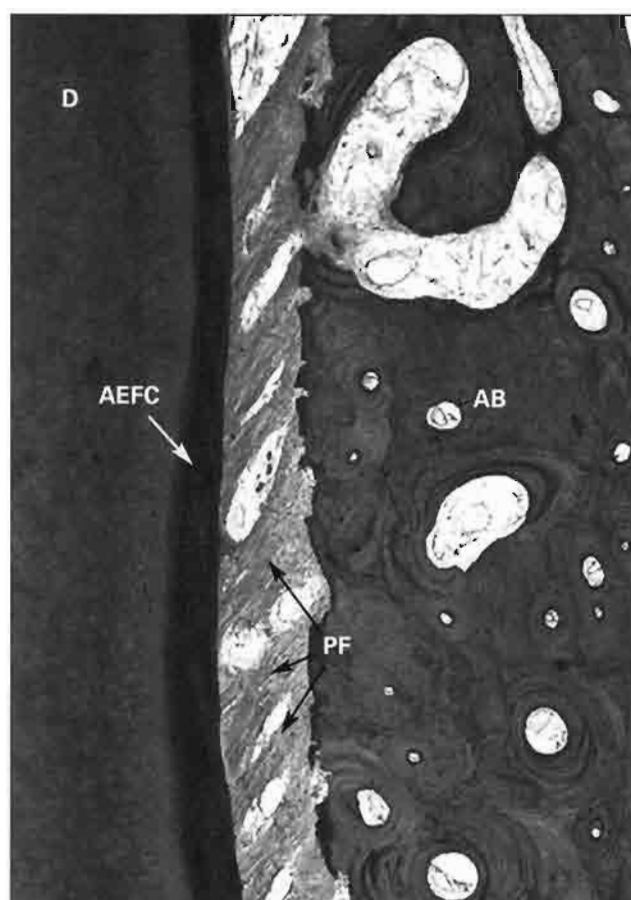


Fig 6-3 Histologic section through the coronal half of the periodontal ligament, depicting alveolar bone (AB), acellular extrinsic fiber cementum (AEFC), dentin (D), and the oblique principal fiber (PF) bundles of the periodontal ligament. (Hematoxylin-eosin stain. Original magnification $\times 100$.)

fibers) at their insertion in newly formed acellular extrinsic fiber cementum and bone and, in the case of fully developed specimens, on a reversal line deep within cementum or bone. Immunocytochemical studies have shown that osteopontin is a significant component of the matrix of the reversal line.

In humans, the PDL has an average width of 0.25 mm; it is wider at the alveolar crest and narrowest at the fulcrum. Decreased functional loads have a tendency to decrease the width of the PDL. The width of the PDL tends to decrease with age. The magnitude of age-related changes is small, and there is considerable variation among subjects.

Approximately 70% of the volume of the ligament is occupied by dense connective tissue (cells and matrix of the principal fiber system), and the remaining 30% is accounted for by loose connective tissue surrounding blood vessels, lymphatic system, and nerves. The balance between these two "compartments" is altered in inflammation. As increasing numbers of inflammatory cells enter perivascular loose connective tissue, it expands by degradation of the adjacent dense connective tissue.

The PDL can be subdivided into three regions:

1. A bone-related region, rich in cells and blood vessels.
2. A cementum-related region, characterized by dense, well-ordered collagen bundles.
3. A middle zone containing fewer cells and thinner collagen fibrils.¹³

It was proposed that the middle zone contained an intermediate plexus, a region of collagen fiber splicing and unsplicing designed to accommodate minor physiologic tooth movement. The concept of an intermediate plexus is not supported by recent studies of collagen synthesis and fibrillogenesis (see "Fibrillogenesis and assembly of collagen fiber networks," later in this chapter). Evidence supports synthesis and turnover of collagen across the entire width of the ligament.^{14,15}

An intermediate plexus-like structure may exist in the PDL of continuously developing incisors of the rat, where there is an abrupt demarcation between a cementum-related zone and a bone-related zone.¹⁶ In this continuously erupting tooth, the cementum-related zone erupts with the tooth, creating a narrow middle band within which collagen fibers are exposed to shearing forces. Ultrastructural studies have shown that the fibroblasts in this slippage plane contain increased amounts of intracellular collagen fibrils, which represent evidence of increased collagen degradation within the lysosomal system.

Components of the Extracellular Matrix

Collagens

Collagen is the most abundant protein of ECM of the periodontal ligament.^{17,18} Type I collagen accounts for approximately 80% of the total collagen content.¹⁹ It is the major component of the principal fibers. Type III collagen, accounting for about 15%

of the total collagen protein, is preferentially localized in reticular fibers located around blood vessels and peripheral nerves. Type III collagen molecules also precipitate with type I molecules during fibrillogenesis to form heterotypic striated fibrils. Immunocytochemical studies have confirmed that type III collagen is associated with type I collagen in the principal fibers.²⁰

Type IV collagen forms the major fraction of basal lamina protein of the blood vessels and nerves in the PDL. Collagen types V and VI form a minor fraction of the PDL collagen. Type V is believed to be associated with the cell surface and to coat larger type III and type I fibrils. Type VI has been shown to be part of the microfibrillar component of oxytalan fibers and may also take part in forming fibrils that serve to attach structures of the basal lamina to the adjacent ECM.²¹ Recent *in vitro* studies have shown that type VI collagen stimulates fibroblast proliferation in a non-integrin-mediated pathway.²²

Immunocytochemical studies have also localized collagen types XII and XIV in the PDL.²³ Type XII, a member of the fibril-associated collagens, forms small fibrils that have a role in the organization of the network of larger collagen fibrils.

Glycosaminoglycans and proteoglycans

Glycosaminoglycans are polymers of repeating disaccharide units constructed of hexosamine and carboxylate or sulfate ester. Glycosaminoglycan chains link covalently with core proteins to form proteoglycans. Structural variations introduced by differences in the amino acid composition of the core protein and in the number, length, and sugar composition of the glycosaminoglycan side chains lead to a wide variety of proteoglycans.

The amino acid sequences of several core proteins have been determined and assigned specific names. Cartilage and vascular interstitial spaces contain very large proteoglycans (aggrecan and versican, respectively). These macromolecules are specialized to resist compressive forces. General connective tissues, such as that of the skin, contain smaller proteoglycans, decorin and biglycan, whose glycosaminoglycans are constructed of repeating units of *N*-acetylgalactosamine and glucuronic acid. These disaccharides can undergo sulfation of the *N*-acetylgalactosamine residues at the 4 or 6 carbon to produce chondroitin-4-sulfate and chondroitin-6-sulfate. Epimerization of glucuronic acid at carbon 5

converts D-glucuronic acid to L-iduronic acid, giving rise to the dermatan sulfate disaccharide unit.

Decorin contains only one glycosaminoglycan side chain of either chondroitin sulfate or dermatan sulfate covalently bound to serine near the amino terminus of the core protein. Biglycan contains two side chains of either chondroitin sulfate or dermatan sulfate. Dermatan sulfate is the predominant repeating disaccharide of skin. Decorin and biglycan core proteins are rich in the amino acids leucine and aspartic acid. Decorin is closely associated with collagen fibrils and is believed to have a function in regulating collagen fibrillogenesis. Decorin also increases the tensile strength of collagen fibrils.²⁴ Biglycan is localized to cell surfaces and pericellular matrices. Its main function may be to regulate the hydration of the extracellular spaces between the collagen bundles.

The proteoglycans of the PDL and gingiva are rich in leucine and aspartic acid, similar to decorin and versican isolated from skin connective tissue. The major class of proteoglycan identified in the PDL is dermatan sulfate. Hyaluronate, chondroitin sulfates, and heparan sulfates are also present.²⁵ The proteodermatan sulfates of the PDL appear related, or perhaps identical, to decorin of the dermis. The chondroitin sulfate fraction is believed to occupy spaces between collagen fibers and to be in part responsible for generating the tissue osmotic pressure of the PDL. This form of proteoglycan may be related to the biglycan of dermal connective tissue. The chondroitin sulfate-rich proteoglycans of the PDL may play an essential role in absorbing compressive shocks and thereby protect the cells of the ligament from damage during occlusal contact.

Ultrastructural studies of the distribution of glycosaminoglycans in the PDL suggest that they are associated with the surface of collagen fibrils.²⁶ Electron microscopic observations of quick-frozen, deep-etched PDL have revealed short, 10-nm, rodlike structures connecting adjacent collagen fibrils and bridging the gap between the fibroblast cell membrane and nearby collagen fibrils. Histochemical staining with alcian blue, a dye with affinity for glycosaminoglycans, has revealed the proteoglycan nature of the rodlike structures.

Hyaluronic acid is a glycosaminoglycan made up of repeating units of *N*-acetylglucosamine and glucuronic acid. It forms very long chains, up to 25,000 disaccharides, but does not associate with a core protein. Because of its large size and polyanionic nature, it occupies a large hydrated domain in the extracellular space. As a result of its high negative charge and large volume, hyaluronic acid regulates

the permeability of the extracellular environment to other molecules.

Hyaluronic acid is present in high concentrations in embryonic tissue, including the developing tooth and PDL. Tissues rich in hyaluronic acid provide pathways of cell migration during embryogenesis. As development progresses, the concentration of hyaluronic acid in the PDL decreases and the levels of dermatan sulfate- and chondroitin sulfate-rich proteoglycans increase.

Oxytalan fibers

Oxytalan fibers are immature elastic fibers consisting of a microfibrillar component of type VI collagen and small amounts of elastin.²¹ Most studies of oxytalan fibers have been of a structural and histochemical nature.^{27,28} Oxytalan fibers arise (or terminate) from the surface of bone and cementum and course in an apicocoronal direction parallel to, and in close apposition to, blood vessels and nerve bundles.^{3,21} Oxytalan constitutes only a minor percentage of the total protein of the PDL.

Noncollagenous proteins

In comparison to collagen and proteoglycans, the noncollagenous proteins occur in small amounts in the PDL. The adhesion molecules, fibronectin, tenascin, and vitronectin, are among the glycoproteins found in the PDL.²⁹ The structure and function of fibronectin and tenascin are described in chapter 1. Fibronectin is widely distributed in the PDL, while tenascin appears to be concentrated at the surface of mineralized cementum and bone.^{30,31} Tenascin is also found in high concentration in granulation tissue as well as in the stroma of oral stratified squamous carcinomas.

Vitronectin is an attachment factor associated with elastic fibers in loose connective tissue. It has been localized throughout the PDL, including in cells lining cementum and bone surfaces. Vitronectin participates in the regulation of blood coagulation, plasminogen activation, and fibrinolysis.

It is now well established that fibroblasts interact with the extracellular matrix through receptor-ligand interactions. Many of the matrix ligands are noncollagenous proteins, such as extracellular adhesion factors.³² For example, it has been shown that the binding of a fibronectin fragment to the $\alpha 5 \beta 1$ fibronectin receptor of rabbit synovial fibroblasts leads to secretion of collagenase.^{33,34} In contrast, when $\alpha 5 \beta 1$ receptors are occupied by intact fibronectin mole-

cules, no collagenolytic response is observed. Periodontal ligament fibroblasts react in a similar way to fibronectin fragments by increasing the expression of collagenase and stromelysin. It is increasingly apparent that receptor-matrix informational exchange is involved in regulating fibroblast connective tissue remodeling, especially in regard to removal of specific collagen fibrils through the cytoplasmic (endocytosis-lysosomal) pathway in gingiva and the PDL.

Fibroblasts

Fibroblasts are the most abundant cells in the PDL. To fully understand the structure and function of the PDL, one must study the biology of the fibroblast, especially its interaction with the extracellular matrix and its response to cytokines and growth factors.

The fibroblastic cell type and its products, the molecules of the extracellular matrix, are among the oldest and most conserved structures of multicellular organisms. Not all fibroblasts are exactly alike. Most PDL fibroblasts are similar to those in the dermis, ie, primarily connective tissue matrix producers, with the exception that PDL fibroblasts have a neural crest origin. A subpopulation of osteoblast-like fibroblasts, rich in alkaline phosphatase, have been identified in the PDL.³⁵⁻³⁷ These cells have the capacity to give rise to bone cells and cementoblasts. They are also responsible for the production of acellular extrinsic fiber cementum in the mature PDL.³⁸

Periodontal ligament fibroblasts are also needed to maintain the normal width of the PDL by preventing the encroachment of bone and cementum into the PDL space.³⁹ The identity of the factors responsible for this activity have yet to be identified.

Structure

Synthetic activity and adhesive interaction with the surrounding extracellular matrix determine the shape of a fibroblast. Progenitor fibroblasts are smaller, less polarized, and contain less rough endoplasmic reticulum (RER) and fewer Golgi saccules.⁴⁰ When grown in vitro on flat surfaces, fibroblasts in nonconfluent cultures assume a well-spread, flattened shape, presenting a triangular profile when viewed from above. Cytoplasmic polarity is evident; the nucleus is located in the narrow end and the Golgi complex faces the broad end of the cell.

In three-dimensional matrices such as collagen gels, or in connective tissues in vivo, the fibroblast assumes a more complex shape, reflecting its contact with a matrix substratum on many of its surfaces.

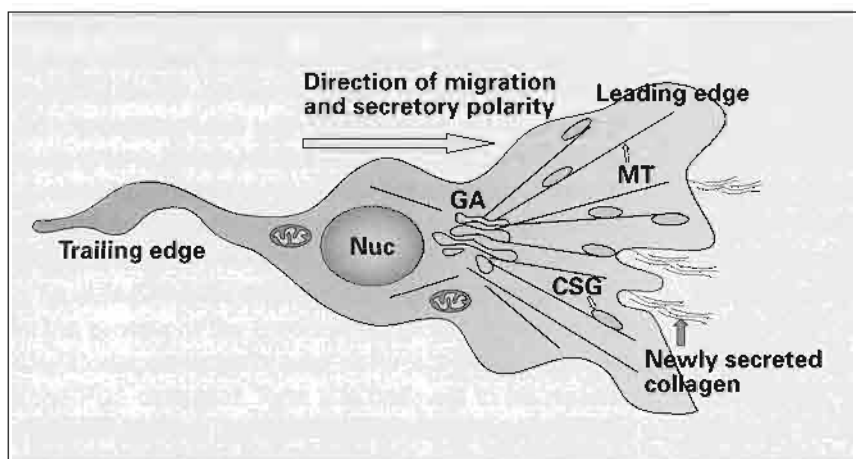


Fig 6-4 Periodontal ligament fibroblast depicting the polarity of cytoplasmic organelles and the close relationship of the cell to collagen fibers. The secretory surface is also the leading edge of the cell. (CSG) Collagen secretion granule; (GA) Golgi apparatus; (MT) microtubule; (Nuc) nucleus.

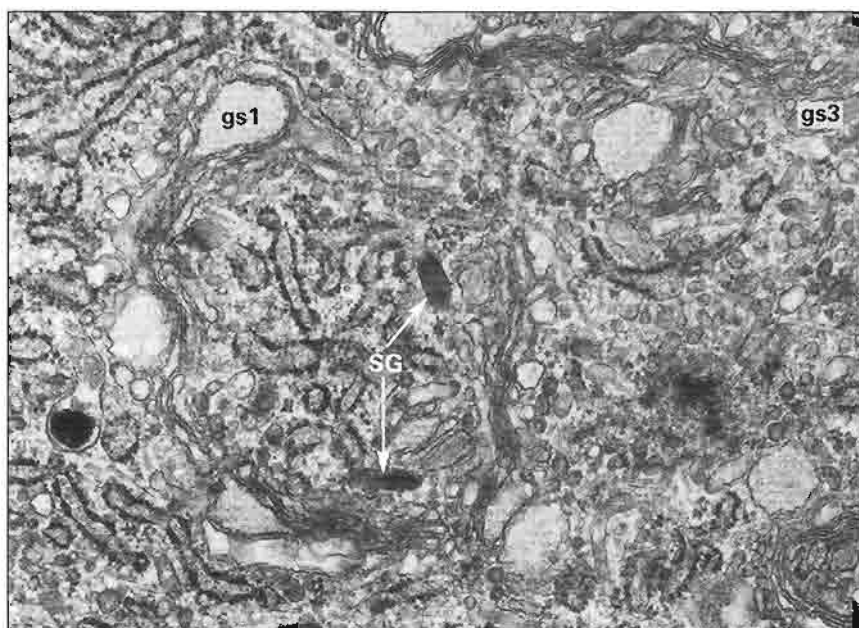


Fig 6-5 Golgi complex of a periodontal ligament fibroblast depicting formation of collagenous matrix secretion granules (SG). Terminal saccules of Golgi cisternae accumulate fibrillar secretory product. Saccules (gs1) attached to cis elements of the Golgi contain a fine, loosely organized content. More mature saccules (gs3) associated with cisternae of the trans-Golgi network demonstrate a more condensed and longitudinally aligned content of collagen. (Original magnification $\times 23,000$.)

Numerous cell processes extend into the spaces between collagen fiber bundles. Macula adherens and gap junctions are made between the cell processes of neighboring fibroblasts.⁴¹

Synthetically active fibroblasts are larger, more elongated, and more polarized than are inactive fibroblasts. A clear distinction in these two extremes is exemplified by dermal fibroblasts activated during wound healing and resting fibroblasts of mature dermal tissues. Most PDL fibroblasts are highly active cells, exhibiting an elongated, well-polarized cytoplasm with extensive areas of contact to collagen fibers (Figs 6-4 and 6-5).^{42,43} Exceptions are noted around blood vessels and near the surface of the ce-

mentum, where fibroblast-like cells appear smaller and less active.

Most fibroblasts in the PDL contain large amounts of RER and well-developed Golgi complexes, indicative of a high rate of protein synthesis.^{16,44} The Golgi complex of the PDL fibroblast contains several Golgi stacks, composed of cisternae and terminal saccules. Each Golgi stack is made up of five cisternae, about 2 μm in length, terminating at each end in an expanded saccule (Fig 6-6).⁴⁵ Immature cisternae at the cis surface of the Golgi complex are slightly dilated and in routine preparations devoid of any stainable content. The saccules associated with these cisternae contain fine, loosely arranged filaments.

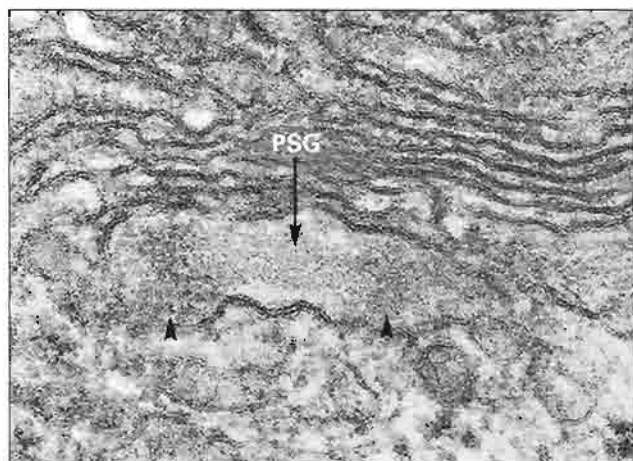


Fig 6-6a Presecretory granule (PSG) developing at the trans-Golgi surface. Note the terminal globular domains (arrowheads) of the fibrillar content. (Original magnification $\times 90,000$.)

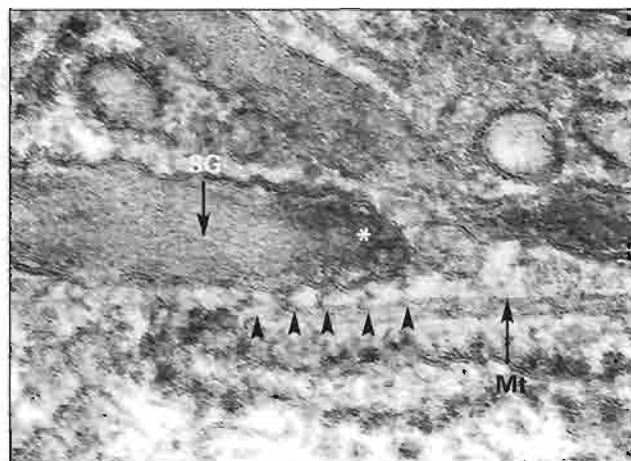


Fig 6-6b Association of mature collagen secretory granule (SG) to a microtubule (Mt) via bridgelike structures (arrowheads). (*) Procollagen terminal domains. (Original magnification $\times 90,000$.)

Coated vesicles are routinely seen in association with the surface of these saccules, suggesting that they are condensing vacuoles. The cisternae of the trans surface contain dense material, and their associated saccules contain rodlike structures with globular terminal elements, resembling segment long-spacing collagen aggregates. These saccules are released to form presecretory granules that quickly associate to microtubules.¹⁴

Autoradiographic studies of the incorporation and secretion of tritiated amino acids, such as proline and glycine, as well as biochemical studies, have confirmed a high rate of protein secretion in the PDL.^{14,18,46} Proline is incorporated into collagen polypeptides in the RER of PDL fibroblasts within minutes of its exit from the bloodstream. At 10 minutes, newly synthesized procollagen molecules are present inside Golgi vesicles and by 20 minutes are ready for secretion within secretory granules associated with microtubules. In less than 30 minutes, newly synthesized collagen fibrils are present in the immediate extracellular vicinity of fibroblasts (Figs 6-7 and 6-8).¹⁴ At 1 hour, newly secreted collagen fibrils are heavily labeled with tritiated proline (see Fig 6-8).

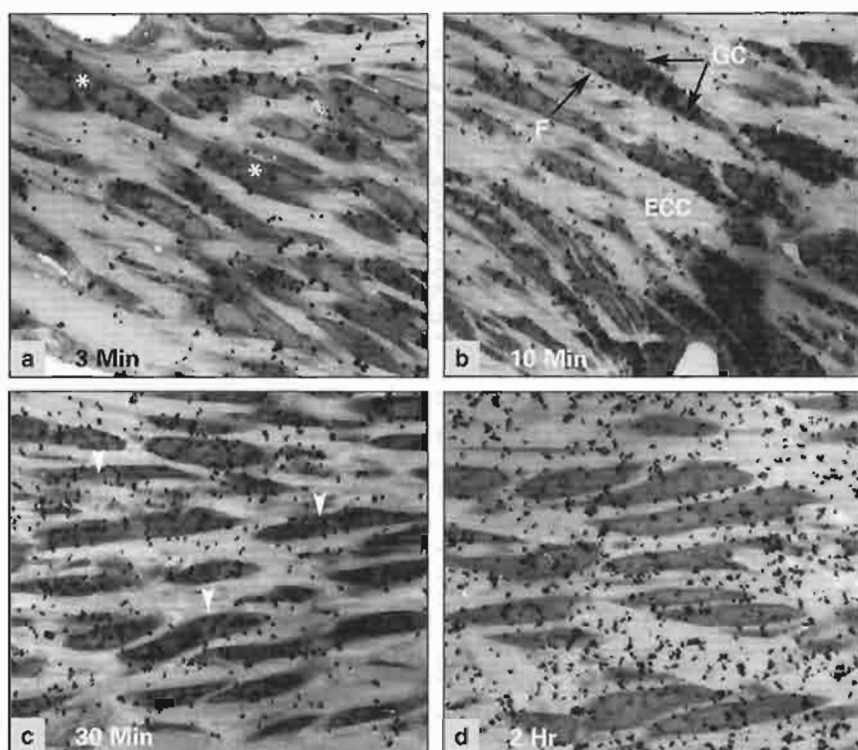
An intact microtubular network is required for movement of collagen secretion granules from the trans-Golgi network to the secretory pole of the cell.^{14,45} During transmigration, the secretory pole of the PDL fibroblast is also its leading edge.

The anatomic and functional polarization of fibroblasts in the PDL was clearly established in ani-

mals fed a diet containing β -aminopropionitrile, a substance that blocks the formation of intermolecular cross-links in the collagen molecules. Autoradiographic analysis of collagen secretion in the PDL of these animals showed that secretion of new (tritiated-labeled) collagen occurred from the end of the cell that was also the leading edge. The secretion of extracellular matrix at the leading edge of a transmigrating cell may have significance in modeling the construction of three-dimensional fiber networks. Chemotactic migration of matrix-forming cells toward bone and cementum could establish the orientation of fringe fibers during development and subsequent repair of the PDL.

Periodontal ligament fibroblasts contain well-defined actin filament bundles in the cortical cytoplasm. They also contain smooth-muscle actin and smooth-muscle myosin, which participate in the formation of cytoplasmic contractile bundles or stress fibers.^{47,48} The stress fibers are oriented parallel to the cell's long axis and terminate at the cell surface at special attachment plaques (fibrinexus). Highly developed stress fibers have been described in fibroblasts of the transseptal fibers.⁴⁹ The presence of actin networks and stress fibers endows the PDL fibroblast with a high degree of contractility, with which it can exert tractional forces on the extracellular matrix (see "Actin filaments," later in this chapter).^{47,48,50}

The mechanical strength of the PDL is dependent on continued stimulation from occlusal contacts. In hypofunctional teeth, the PDL undergoes atrophy.⁵¹ Mechanical stretching of PDL fibroblasts stimulates



Figs 6-7a to 6-7d Light microscopic autoradiographs depicting the localization of tritiated proline. (Original magnification $\times 800$.)

Fig 6-7a At 3 minutes, most of the proline is located over the extracellular space or at the cell periphery. (*) Pair of migrating daughter fibroblasts.

Fig 6-7b At 10 minutes, the rough endoplasmic reticulum and Golgi complex (GC) are labeled. (F) Fibroblast; (ECC) extracellular collagen.

Fig 6-7c At 30 minutes, the Golgi complex (arrowheads), cell processes of the secretory pole, and the extracellular space are labeled.

Fig 6-7d At 2 hours, most of the proline is incorporated in the extracellular collagen matrix.

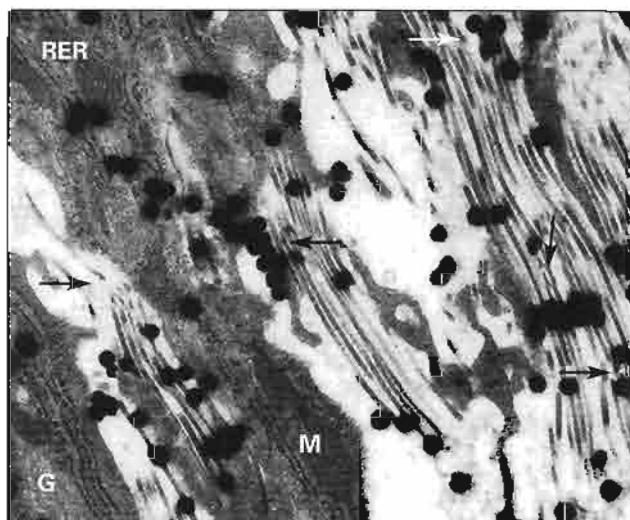


Fig 6-8 Electron microscopic autoradiograph depicting silver grains (arrows) over newly secreted collagen 1 hour after tritiated proline injection. (G) Golgi complex; (M) mitochondria; (RER) rough endoplasmic reticulum. (Original magnification $\times 13,000$.)

their level of activity. Stretched fibroblasts upregulate several small cytoplasmic guanosine triphosphate-binding proteins that function in secretory transport pathways.⁵²

Functions

Collagen synthesis and secretion occur across the entire width of the PDL.^{15,53} This suggests that adaptation of the principal fibers to the physiologic re-

quirements of tooth movements (as in medial drift or continued eruption) occurs by collagen phagocytosis, and deposition and incorporation of new collagen, rather than by "unsplicing and splicing" of collagen fibers.⁵³ The concept of an intermediate plexus, a middle zone of unsplicing and splicing of collagen fibers, is regarded to be an oversimplification of the physiologic adaptation of the PDL (see "Fibrillogenesis and assembly of collagen fiber net-

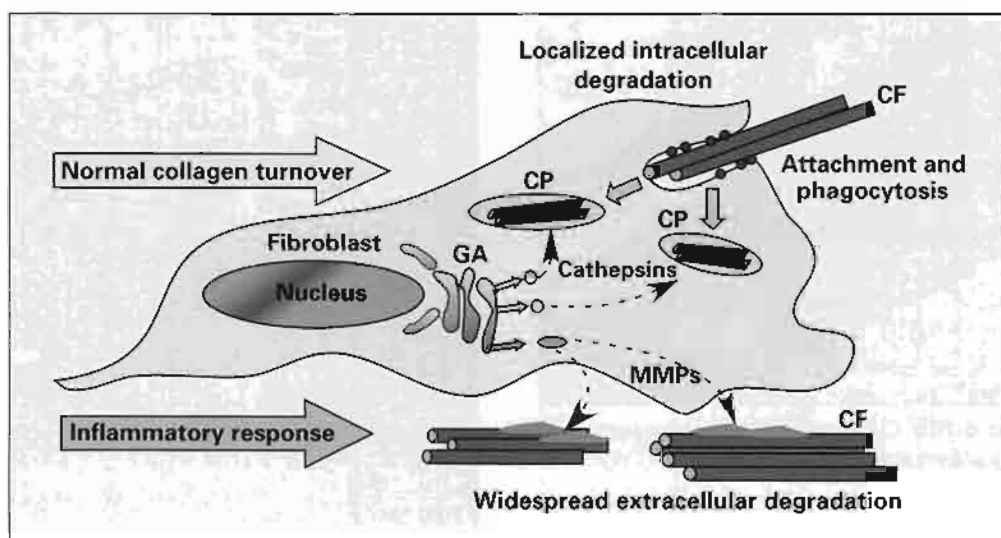


Fig 6-9 Degradation of collagen fibrils (CF) via the intracellular and extracellular pathways. In the intracellular pathway, collagen fibrils are captured by integrins and membrane metalloproteinases (red dots) and subsequently internalized into collagen phagosomes (CP), where they are degraded by cathepsins transported from the Golgi apparatus (GA). This pathway is responsible for localized and physiologic collagen removal. In the extracellular pathway, collagen is degraded outside the cell by secreted matrix metalloproteinases (MMPs). The external pathway causes rapid and widespread collagen destruction, such as in an inflammatory reaction. How these pathways are activated and regulated remains poorly understood. (Based on the report of Everts et al.⁶²)

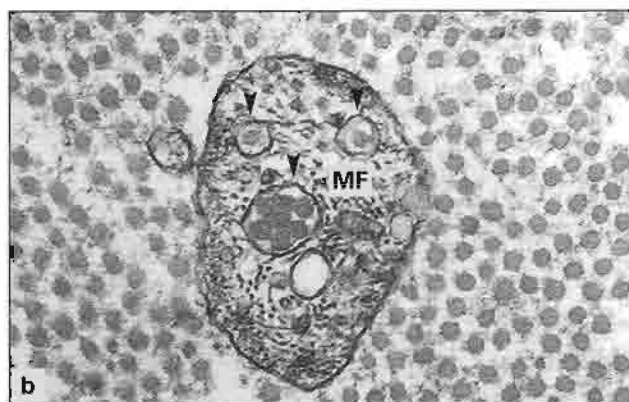
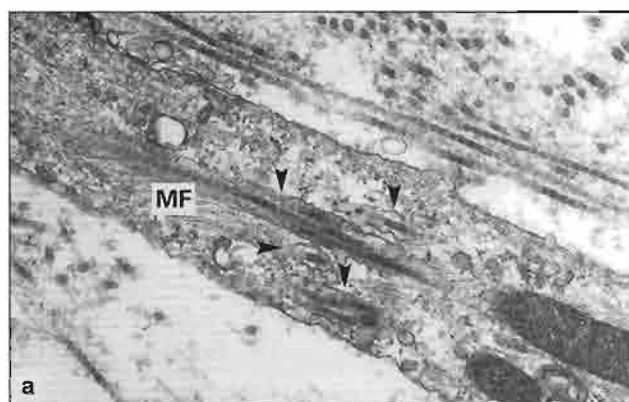
works"). Collagen metabolism is believed to vary from one principal fiber bundle to another in response to local stimuli.^{15,46} For example, the dentoalveolar fibers turn over more rapidly than do the transseptal fibers. The molecular aspects of collagen synthesis and fibril formation are discussed later in this chapter.

The fibroblast is not only responsible for the formation of collagen fiber networks but is also involved in the removal of collagen fibrils (Fig 6-9).^{3,54-56} With the advent of electron microscopy, striated collagen fibrils were observed inside vesicles of fibroblasts, particularly abundant in the PDL fibroblasts (Fig 6-10).⁵⁵⁻⁵⁷ Localization of acid phosphatase inside the same vesicles that contained intracellular collagen fibrils added support to the idea that fibroblasts are involved in lysosomal digestion of collagen fibrils.^{56,58} In vitro studies have demonstrated that fibroblasts are eminently capable of phagocytosing collagen fibrils from the extracellular environment and degrading them inside phagolysosomal bodies.^{3,59}

Additional study of this activity indicates that collagenase (matrix metalloproteinase 1 [MMP-1]) is not involved in the intracellular phase of the degradation of collagen fibrils.⁶⁰ Lysosomal cysteine proteinases

(cathepsins B, L, and N) of the lysosomal granules are capable of rapid degradation of internalized collagen fibrils (see Fig 6-9). It has been suggested that cell surface MMPs and integrin collagen receptors localized in phagocytic clefts (see Fig 6-9) may regulate the initial steps in fibril internalization.^{61,62} Interference with focal adhesion kinase may reduce the bond strength between integrins and collagen. Plasma membrane alkaline phosphatase has also been implicated in promoting collagen phagocytosis, probably through its ability to bind collagen.⁶³

Recent studies have provided convincing arguments that the intracellular pathway of collagen degradation is responsible for the physiologic turnover of collagen in the PDL (which is measured to be among the highest in the body).^{3,64} It is estimated that the half-life of phagocytosed collagen fibrils is about 30 minutes in the PDL of the rat. The extracellular pathway, involving collagenase, is reserved for large-scale indiscriminate removal of collagen fibers, such as in inflammation. In physiologic remodeling of connective tissues, where selective replacement of collagen fibrils is required, a more controlled enzymatic attack is necessary. In this case, the intracellular phagocytic pathway is used.



Figs 6-10a and 6-10b Cytoplasmic vacuoles containing collagen fibrils (arrowheads) in longitudinal (a) and cross (b) sections. The adjacent cytoplasm is rich in microfilaments (MF). (Original magnification $\times 34,000$ [a], $\times 60,000$ [b].)

In vitro studies of PDL fibroblasts have shown a positive correlation between cellular aging and collagen phagocytosis. In addition, older cells are characterized by higher levels of lysosomal enzymes, less alkaline phosphatase, and lower rates of collagen secretion.⁶⁵ These results support the theory that the net loss of collagen observed in older PDLs could be caused by an excess of resorption over the production of new collagen.

Cells of the osteoblastic subtype can be identified by their high level of alkaline phosphatase and by their ability to bind a newly discovered cementum attachment protein.⁶⁶ Cells of this subtype produce mineralized nodules in vitro while maintaining a fibroblast morphotype. Analysis of the mineralized matrix has revealed the presence of osteopontin and bone sialoprotein, characteristics shared with osteoblasts and cementoblasts.⁶⁶ Transmission electron microscopy of these mineralized nodules reveals a tissue with similarities to acellular extrinsic fiber cementum. These results demonstrate the important role of PDL fibroblasts in anchoring collagen fibrils to the mineralized matrix of the root surface.

The PDL's potential for osteoblastic differentiation must perform at a high level during new bone formation in the repair of extraction sockets.⁶⁷ Proliferation of fibroblasts contained in the remnants of the PDL that remain anchored to the alveolar bone migrate into the bone socket soon after tooth extraction. Within days these cells differentiate into osteoblasts and form trabeculae of new bone.

The osteoblastic characteristics of PDL fibroblasts are downregulated by IL-1 β . The phenotypic change induced by IL-1 β also increases responsiveness to lipopolysaccharide.^{68,69} Periodontal ligament fibroblasts treated with IL-1 β respond to lipopolysaccharide by expressing proinflammatory cytokines such as IL-6, IL-8, and tumor necrosis factor α .⁶⁸ Interleukin 1 β and tumor necrosis factor α act synergistically to stimulate the expression of monocyte chemoattractant protein 1, a potent chemokine specific for cells of the monocyte and macrophage lineage.⁷⁰

Monocytes and macrophages

Cells of the monocyte and macrophage lineage are normal inhabitants of the PDL. These cells are typically found in the perivascular and perineural cuffs of loose connective tissue and are rarely observed to reside in the dense connective tissue of the principal fiber bundles. In light microscopic sections, monocytes appear round to oval in outline and are characterized by a finely ruffled surface. At the ultrastructural level, the monocyte cell surface is observed to have many microvilli and folds and to give rise to many coated vesicles. Dense lysosomal granules are characteristic features of the cytoplasm. Activated monocytes contain a larger Golgi complex, more RER, a more highly ruffled cell surface, and cytoplasmic polarity. Macrophages, arising by the maturation of activated monocytes, are typically larger and contain numerous phagolysosomal inclusions.

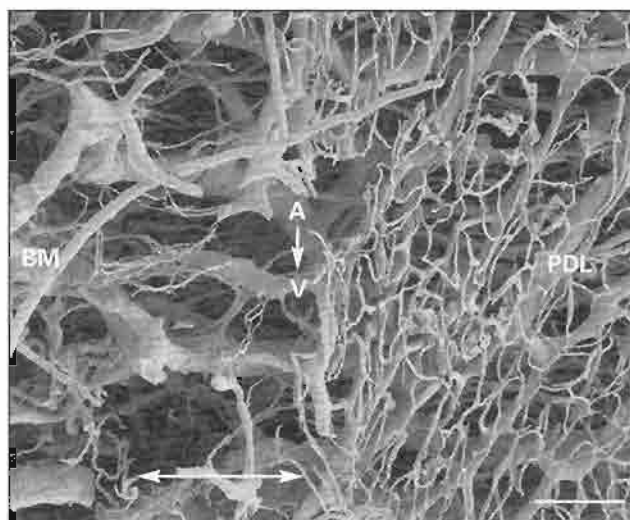


Fig 6-11a Resin cast of the vascular plexus of the periodontal ligament (PDL). Veins (V) and arterioles (A) communicate from the bone marrow (BM) to the PDL through Volkmann's canals in the alveolar bone plate (A-V and double-headed arrow). Note the meshwork of capillaries in the PDL. (Reprinted from Matsuo and Takahashi⁷¹ with permission from John Wiley & Sons.)

Monocytes exit blood vessels to enter the PDL in response to chemotactic stimuli. The nature of the chemotactic factors that are responsible for attracting monocytes to exit blood vessels of apparently healthy PDL are unknown. During inflammation of the PDL, IL-1 β and tumor necrosis factor α contribute to monocyte infiltration by stimulating fibroblasts to secrete macrophage chemoattractant protein 1. Numerous substances, including endotoxin, immune complexes, and lymphokines, can activate monocytes. Activated monocytes secrete collagenase, elastase, plasminogen activator (PA), and lysosomal hydrolases and are thus highly capable of degrading extracellular matrices (see "Fibrillogenesis and assembly of collagen fiber networks"). Electron microscopic studies indicate that the degradative process is most efficient in the immediate pericellular space of the monocyte.

In view of the known capability of monocytes and macrophages to degrade extracellular matrices, it is logical to suspect that they are partly responsible for establishing the zone of loose connective tissue around the blood vessels of the PDL. A physiologic balance between the levels of loose (areolar) connective tissue and dense fiber bundles must be maintained to ensure firm attachment of the tooth. During inflammation, the zones of loose connective tissue expand with infiltrates of lymphocytes, plasma cells, and neutrophils, at the expense of dense connective tissue.

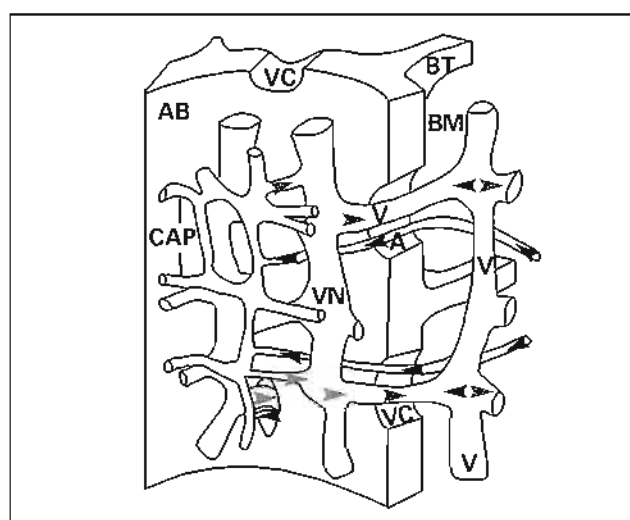


Fig 6-11b Polygonal capillary (CAP) network of the periodontal ligament and its anastomosis with vessels in the bone marrow (BM). (A) Arterioles; (AB) alveolar bone; (BT) bone trabeculae; (V) veins; (VN) venular network; (VC) Volkmann's canal. (Reprinted from Matsuo and Takahashi⁷¹ with permission from John Wiley & Sons.)

Supply of Blood to the Periodontal Ligament

There are three avenues of blood supply to the periodontal tissues:

1. Branches of the suprapariosteal vessels that supply the gingiva anastomose with venules in the coronal part of the PDL, just apical to the termination of the junctional epithelium.
2. Dental arterioles from the maxillary and mandibular alveolar arteries that course coronally in the PDL to supply mainly its apical part.
3. Branches of the interdental septal arteriole that penetrate the alveolar wall and contribute to a capillary and postcapillary plexus in the middle of the PDL.⁷¹

Arterial vessels leaving the bone marrow pass through Volkmann's canals in the alveolar plate. On entering the PDL, the arterial vessels give rise to a capillary plexus near the root surface and a postcapillary venous plexus closer to the bone surface before reentering the bone marrow via draining venules (Fig 6-11).⁷¹ When occlusal pressure is placed on a tooth, blood is forced out of the PDL vascular plexus back to larger veins in the bone marrow. In this manner, the vascular plexus acts as a shock absorber to help cushion the forces generated during mastication.⁷¹

Innervation of the Periodontal Ligament

Peripheral branches of the trigeminal nerve enter the ligament through the apical foramen and from lateral openings in the alveolar wall. These branches terminate in the connective tissue as free endings or as part of complex sensory units.^{72,73} The free nerve endings are believed to transmit pain sensations. The complex sensory terminals respond to slight deformations of the ligament, providing afferent information critical to coordination of muscular activity for chewing as well as for the initiation of protective reflexes.⁷⁴

Physiologic data indicate that the principal mechanoreceptors in the PDL are Ruffini-type end organs that act as slowly adapting receptors to tension in the surrounding collagen matrix.⁷⁵ Histochemical studies show high levels of Na⁺-K⁺-adenosine triphosphatase and carbonic anhydrase in Ruffini receptors of the PDL.⁷⁶ The role Ruffini end organs play as PDL mechanoreceptors is described in chapter 10.

Slowly adapting mechanoreceptors respond to small displacements of the teeth, on the order of 2 to 10 μ m, and can be activated by forces as low as 1 to 2 g. The response is characterized by a train of impulses fired for as long as the stimulus is applied; the frequency of discharge is related to the amplitude of the stimulus. These units are also sensitive to the direction in which the tooth is moved, presumably as a result of their position in the ligament.⁷⁷ A loosely defined capsule, partially surrounding branched nerve terminals in close approximation to collagen, characterizes the Ruffini complex.⁷⁸

Receptor end organs with a lamellated capsule similar to that of Meissner's corpuscle have been observed in the apical part of the PDL. Receptors of this type are believed to be rapidly adapting receptors with a high threshold (greater than 10 g) to mechanical stimulation of the teeth. The response is limited to the on and off parts of the stimulus.

Basic Science Correlations

Structure of collagens I and III

Collagen molecules are known to constitute a large family of proteins that share a common helical domain.⁷⁹ Diversity among the 19 different types of collagen resides in smaller nonhelical segments of the molecule. The biology of the connective tissues of

the periodontal ligament have been the subject of in-depth reviews.⁸⁰ Collagens form the predominant protein of the periodontal tissues. Type I and type III collagens constitute roughly 80% and 15%, respectively, of the total collagen of the PDL. They undergo polymerization to form collagen fibrils of relatively uniform diameter in the PDL (Fig 6-12).

The type I collagen molecule is made up of three polypeptide chains (α chains, type I) assembled in a left-handed triple helix. Nascent polypeptide α chains synthesized in the RER consist of five domains: NH₂- and COOH- terminal propeptides, N- and C-telopeptides, and larger α -helical middle segments that participate in the formation of the triple helix (Fig 6-13).⁸¹ The α -helical segment of type I collagen contains 1,014 amino acid residues composed of 338 glycine-X-Y triplets. Proline is most often in the X position and hydroxyproline is most often in the Y position. The regular occurrence of glycine at every third position permits the polypeptide chain to fold and to hydrogen bond with adjacent α chains to form the triple helix. In collagen type I, two α chains similar in amino acid sequence, the $\alpha 1(I)$ chains, interact with a third α chain composed of a different amino acid sequence, the $\alpha 2(I)$ molecule.

Helix formation occurs while the proteins are still in the RER. Disulfide bonds formed between the carboxyl ends of the polypeptide chains stabilize the three chains to allow the helix to form from the COOH terminus to the NH₂ terminus. The helical shape of the collagen molecule protects its peptide bonds from attack by proteolytic enzymes other than the matrix metalloproteinases.

Hydroxylation and glycosylation reactions also occur in the RER to produce posttranslational modifications of some amino acid residues.⁸² About one half of the 200 proline residues are converted to hydroxyproline by the enzyme prolyl hydroxylase. This reaction requires oxygen, α -ketoglutarate, ascorbic acid, and ferrous ion. Lysyl hydroxylase converts lysine to hydroxylysine in a similar process.

Cleavage of the propeptides must occur in the extracellular space before collagen molecules can undergo aggregation to form fibrils. Procollagen peptidases, one specific for removing the propeptide at the NH₂ terminus and one for cleaving the propeptide at the COOH terminus, function in the extracellular space to convert procollagen to collagen. The resulting molecules still contain nonhelical telopeptides at each end. These telopeptides are important in forming intermolecular bonds to stabilize the side-by-side aggregation of collagen molecules into fibrils.

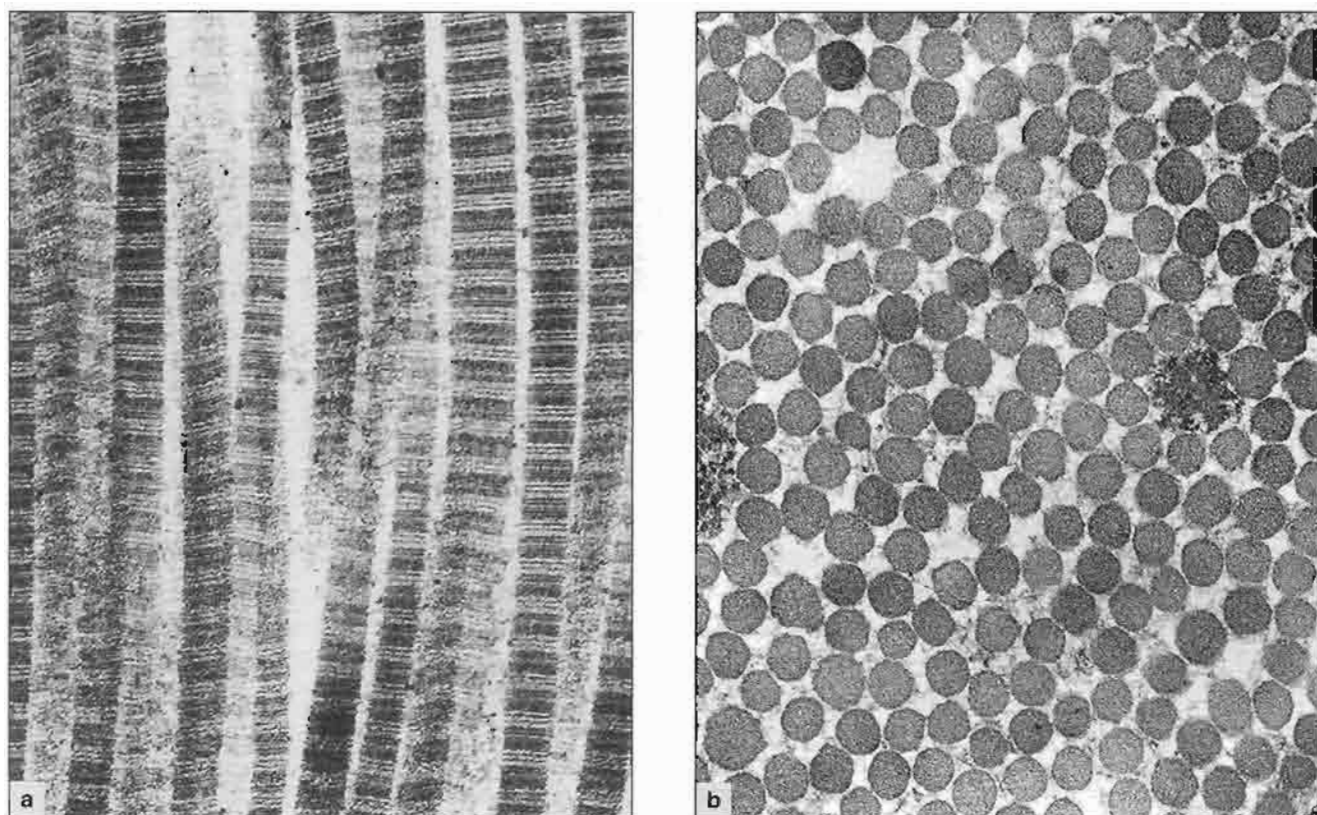


Fig 6-12 Mature collagen fibrils of the periodontal ligament viewed in longitudinal (a) and cross (b) sections. Note the regularity of the fibril diameter. (Uranyl acetate and lead citrate stain. Original magnification $\times 80,000$.)

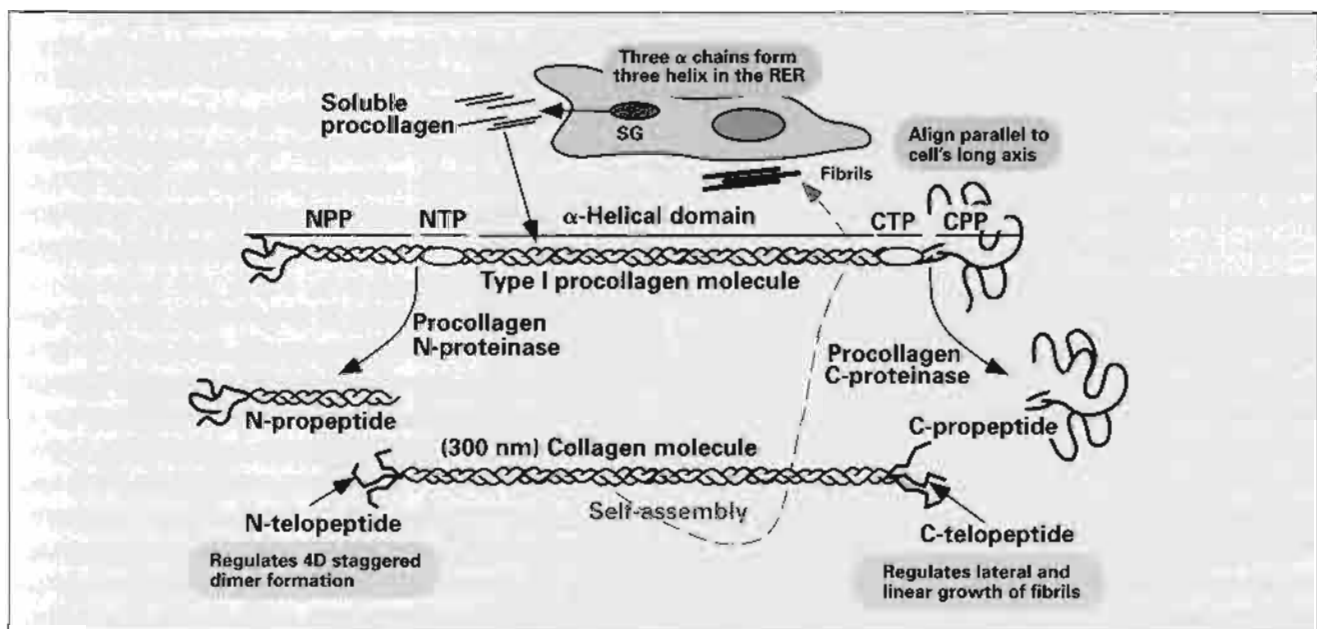


Fig 6-13 Extracellular cleavage of procollagen and assembly of collagen fibrils. Type I procollagen molecule is constructed of three α polypeptide chains coiled together to form a large central helical domain stabilized by disulfide bonds at the COOH terminal. The amino propeptide (NPP) and carboxy propeptide (CPP) are cleaved by procollagen peptidases after the procollagen molecule has been secreted into the extracellular space. The resulting collagen molecules undergo self-assembly to form fibrils. Fibril formation is aided and stabilized by intermolecular interactions at the remaining amino and carboxy nonhelical segments (telopeptides) of the α chains. (CTP) Carboxy telopeptide; (NTP) amino telopeptide; (RER) rough endoplasmic reticulum; (SG) secretory granule; (4D) quarter periodic.

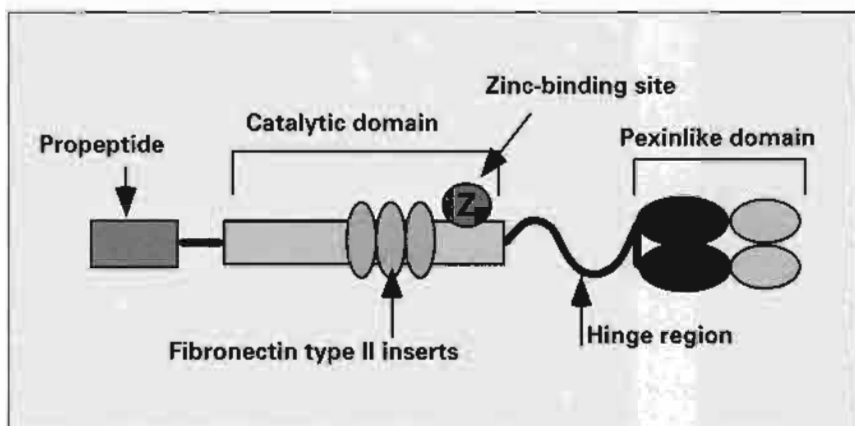


Fig 6-14 Domains of the prototypical gelatinase matrix metalloproteinase structure. The catalytic domain contains a zinc-binding site (Z) that is required in the functional enzyme. The pexinlike domain determines substrate specificity and has been shown to bind to certain cell membrane integrins. (Adapted with permission from Birkedal-Hansen.⁸⁶)

Lateral alignment with a 67-nm overlap, or quarter stagger, of adjacent collagen molecules gives rise to the typical striated appearance of mature fibrils (see Fig 6-13). The quarter stagger arrangement of the collagen molecules creates gaps or "hole" regions in the fibrils. Hole regions are important sites of biomineralization and avenues by which other molecules such as enzymes can gain access to the inner regions of the fibrils (see chapter 8).

The type III collagen molecule contains only one class of α chains. Its formula is written as $\alpha 1(\text{III}) 3$. Unlike other fibril-forming collagens, type III polypeptide chains contain intramolecular disulfide cross-links within the helical segment of the molecule. Immunocytochemical studies have shown that type III collagen is present in small argyrophilic fibrils (reticular fibers). It is also coprecipitated, along with type I molecules, in heterotypic striated collagen fibrils, such as the principal fibers of the PDL.

Fibrillogenesis and assembly of collagen fiber networks

Following the discovery that collagen molecules undergo self-assembly to form fibrils, little attention was focused on the cellular control of connective tissue morphogenesis. During the past decade, evidence has accumulated that fibroblasts not only synthesize and secrete the components of the extracellular matrix but also exert an organizational influence on collagen fibrils during the assembly of the macromolecular matrix.^{24,83} The observation that fibroblasts transmit tension to extracellular collagen fibrils, and the recognition that fibroblasts exhibit anatomic and functional polarity during matrix deposition, provided early clues of an architectural relationship between

cells and matrix. The role of microtubules in transporting, aligning, and positioning collagen secretory granules in parallel alignment to existing extracellular fibrils also pointed to a cellular mechanism of control in fiber-building activities.⁴⁵

Recent studies of tendon fibroblasts have demonstrated that these cells form extracellular microcompartments into which collagen fibrillogenesis takes place.⁶⁴ Alignment of secretory granules parallel to the long axis of these compartments appears to be an essential requirement. New collagen fibrils are assembled close to the cell membrane domain that delimits the compartment. Collagen fibrils are bundled into tightly organized fibers through the lateral association of newly deposited fibrils inside these microcompartments. Observation of cementogenesis and the concurrent development of Sharpey's fibers has revealed that microcompartment formation by PDL fibroblasts may play a role in aligning and packing the collagen fibrils into fibers of uniform diameter at the root surface.

Of additional interest is the finding that collagen fibrils are not indefinite in length. Collagen fibrils of chick tendons have been shown to range in length from 30 to 100 μm . Ultrastructural reconstruction of serially sectioned chick embryo tendons has shown that fibers are assembled by overlapping fibril segments approximately 100 μm in length and tapered at each end.^{83,85} The small proteoglycan decorin appears to hamper collagen fibril assembly in vitro, suggesting that it may have a regulatory role in fibrillogenesis in vivo.⁸⁵ These observations may have important implications for the PDL. It is suggested that collagen fibers, such as those that constitute the principal fibers, are not made up of fibrils that are continuous across the ligament but rather are macroaggregates of relatively short fibril segments.

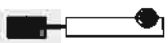
Enzyme domain structure	Enzyme	MMP No.	Substrates
	<i>Gelatinases</i> Gelatinase A Gelatinase B	MMP-2 MMP-9	Denatured collagens; native collagens IV, V, VII, X; elastin, fibronectin
	<i>Collagenases</i> Fibroblast-type Polymorphonuclear neutrophil-type	MMP-1 MMP-8	Collagens I, II, III, VII, VIII, X
	<i>Stromelysins</i> Stromelysin-1 Stromelysin-2 Stromelysin-3 Matrilysin	MMP-3 MMP-10 MMP-11 MMP-12?	Proteoglycan core protein; fibronectin; laminin Collagens IV, V, IX, X; elastin Elastin
		MMP-7	Fibronectin; laminin; collagen IV; proteoglycan core protein

Fig 6-15 Enzyme domain structure, number, and substrate specificities of the known matrix metalloproteinases (MMPs). (Z) Zinc-binding site. (Adapted with permission from Birkedal-Hansen.⁸⁶)

Such an arrangement would appear to permit a greater flexibility for remodeling and reattachment along the entire length of a principal fiber bundle.

Matrix metalloproteinases

The extracellular degradation of collagen and other matrix components is regulated by a complex system of enzymes (MMPs) and enzyme inhibitors.^{86,87} The MMP prototype domain structure consists of a propeptide sequence, a catalytic unit containing a zinc-binding site, and a pexinlike domain hinged to the catalytic unit (Fig 6-14). Nine members of the MMP family are depicted in Fig 6-15. The gelatinases contain fibronectin type II sequences within the catalytic domain. The pexinlike domain determines substrate specificity. All MMPs contain a zinc-binding site in the catalytic domain.

Native type I and type III collagen fibrils, the major components of the periodontal ECM, are degraded by fibroblast-type collagenase (MMP-1) and, during inflammation, by neutrophil-type collagenase (MMP-8) (see Fig 6-15). The expression of MMP-1 and MMP-3 (stromelysin) in human PDL fibroblasts and in monocytes and macrophages is stimulated by IL-1 β and decreased by transforming growth factor β (TGF- β).⁸⁸⁻⁹⁰ Matrix metalloproteinase 1 has the ability to penetrate the triple helix of the native collagen

type I molecule to cleave it into two unequal segments. These segments are then further degraded by gelatinases. Denatured collagen, or gelatin, is collagen that has lost its triple-helix configuration. It can be degraded by gelatinases A and B (MMP-2 and MMP-9).

Other MMPs include the stromelysins and matrilysin (see Fig 6-15). Stromelysins attack proteoglycans, elastin, fibronectin, laminin, and collagen types IV, V, IX, and X. Matrilysin degrades gelatin and the native forms of collagens IV, V, VII, and X.⁸⁶

Matrix metalloproteinases are calcium and zinc dependent. They are secreted as proenzymes (zymogens) that must be activated by proteolytic processing.⁹¹ Several enzymatic pathways are available for extracellular activation of MMPs. Plasmin, a broad-spectrum serine proteinase, is capable of activating collagenase. Plasmin is produced by the cleavage of its precursor, plasminogen, through the action of plasminogen activators (Fig 6-16).

There are two types of PA: tissue PA (tPA) and urokinase PA (uPA). Both are produced by fibroblasts and macrophages. Tissue PA is secreted as a soluble enzyme that can activate collagenase at sites distant from the cell surface. Plasminogen activator inhibitors 1 and 2, secreted by the same cells that make tPA, regulate the amount of active tPA in tissues. Plasminogen activator inhibitor 1 has been

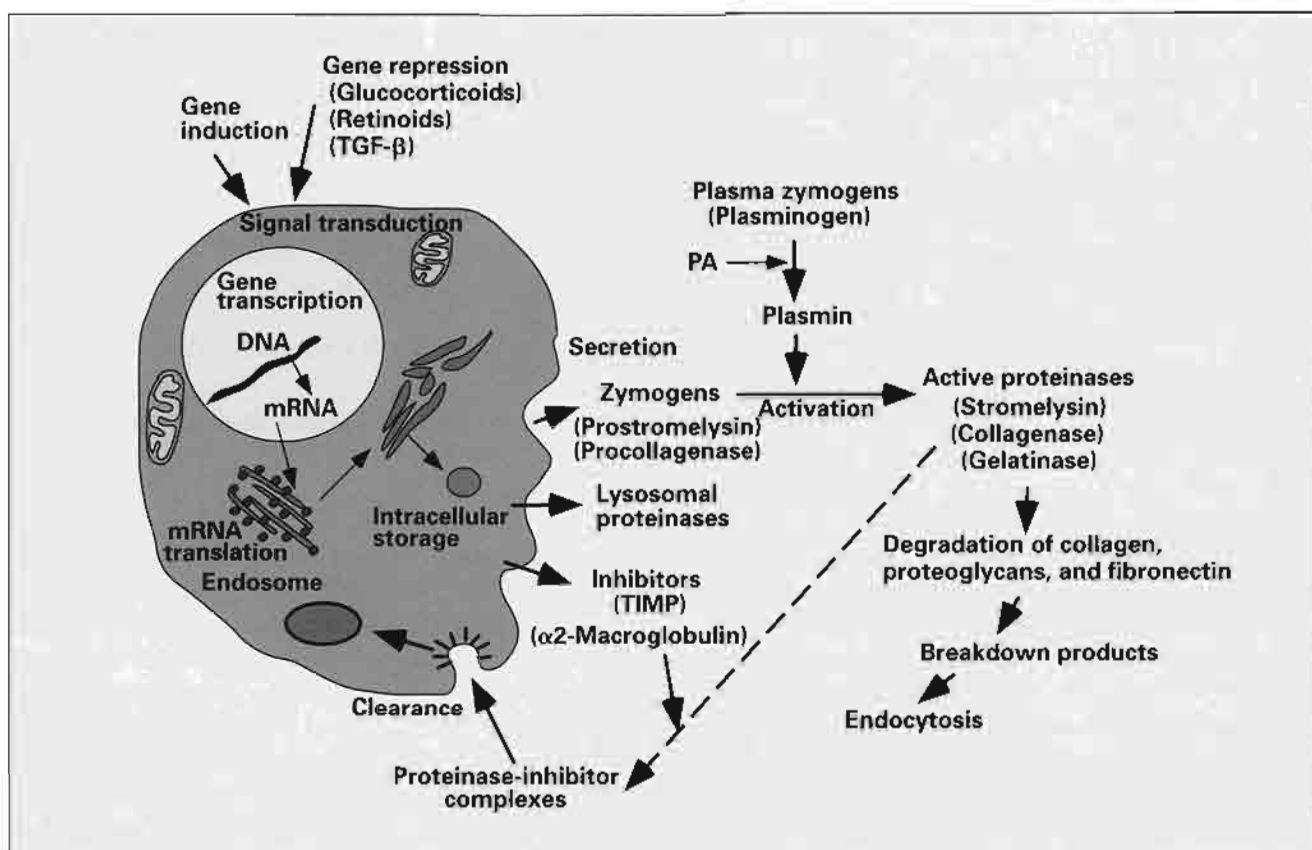


Fig 6-16 Major pathway for activation of matrix metalloproteinases. Prostromelysin and procollagenase are secreted as inactive forms that are proteolytically activated by plasmin. Plasmin is generated from plasminogen by the action of plasminogen activator (PA). Urokinase PA, associated with the cell surface, activates matrix metalloproteinases in the immediate vicinity of the cell. Tissue PA, located at a distance from the cell, is responsible for a more diffuse activation of matrix metalloproteinases. Extracellular matrix breakdown products and matrix metalloproteinase-inhibitor complexes are removed by endocytosis and degraded within lysosomes. (TGF- β) Transforming growth factor β ; (TIMP) tissue inhibitor of matrix metalloproteinase. (Adapted from Werb and Alexander⁹¹ with permission from Elsevier Science.)

shown to be widely distributed in gingival connective tissue, suggesting that it may act as the principal inhibitor of tPA and uPA. The proinflammatory cytokine IL-1 β stimulates the expression of tPA in gingival fibroblasts. This observation could account for the high level of tPA in inflamed gingival connective tissue.

Urokinase PA is localized to segments of the fibroblast cell surface that contact the extracellular matrix. Migrating fibroblasts and mononuclear cells localize uPA at the leading edge of the cell. The presence of MMPs and uPA (or other enzymes that have a role in activating MMPs) at the leading edge of the cell assists in the breakdown of the ECM in advance of cell migration.

In some forms of cancer, the invading cancer cells and the endothelial cells that accompany them bind MMP-2 on the cell surface by association to the integrin α v β 3.⁹² Urokinase PA is localized to the same

site. Activation of MMP-2 at the cell surface, by the ability of uPA to generate plasmin from plasminogen, disrupts the collagenous ECM, thereby facilitating the migration of the cancer cells.

Another source of MMP activation is neutrophil elastase. The saliva and gingival crevicular fluid of patients with periodontitis contain elevated concentrations of neutrophil elastase.

Membrane-type MMPs have recently been identified in certain types of cancer cells. These are single-pass transmembrane proteins with catalytic domains that extend into the extracellular space. Membrane-type 1 MMP has a catalytic domain similar to that of MMP-1 and is capable of splitting native collagen that is adjacent to the cell surface.⁹³

Of additional significance is the observation that membrane-type 1 MMP can also activate MMP-2. Matrix metalloproteinase 2 localizes on the surface of

invasive cells by its association with integrin receptors.⁹² The combined catalytic activity of membrane-type 1 MMP and MMP-2 in the immediate vicinity of the cell surface is advantageous to cell migration and connective tissue invasion. It will be interesting to learn if similar membrane-type MMPs are expressed in PDL fibroblasts and especially as it may apply to collagen phagocytosis.

Inhibitors of MMPs control the degradation of the collagenous ECM. Tissue inhibitors of metalloproteinase block fibroblast-type collagenase and other MMPs (see Fig 6-16). A component of serum, α 2-macroglobulin, is a broad-spectrum MMP inhibitor. Enzyme-inhibitor complexes are taken up by endocytosis and digested in the endosomal-lysosomal apparatus of fibroblasts and macrophages. A disturbance of the balance between the level of active collagenase and tissue inhibitors of metalloproteinase, favoring active enzyme, can lead to tissue destruction.⁹⁴

Matrix metalloproteinases have been implicated in the pathogenesis of periodontal disease.^{86,95,96} MMP-1, MMP-2, MMP-8, and MMP-9 are elevated in tissue and oral fluids of sites affected by periodontitis. The proinflammatory cytokine IL-1 β , present in inflamed tissues, increases the expression of stromelysin 1 (MMP-3) by PDL fibroblasts.⁹⁰ Following therapy, the levels of MMPs decline as inflammation subsides. The MMPs in inflamed connective tissue may also be activated locally by bacterial proteases or by plasmin.

Human gingival epithelial cells produce a factor that stimulates collagenase production by PDL fibroblasts. It has been suggested that this could be a key pathogenic mechanism for the apical migration of the junctional epithelium in periodontitis.⁹⁷ Destruction of the connective tissue attachment directly beneath the apical part of the junctional epithelium uncovers the cementum, providing a substratum for epithelial cell migration.

Actin filaments

Actin is expressed at relatively high levels in PDL fibroblasts. It forms a filamentous network in the cortical cytoplasm of all fibroblasts. Actin filaments associate with myosin to form long bundles or stress fibers in some PDL fibroblasts (Fig 6-17).^{48,49} At least six genetically different actin types are known. Two sarcomeric forms are found in striated and cardiac muscle cells. Two types are present in smooth-muscle cells, and two forms are present in nonmuscle cells. The actins in the nonmuscle cells represent the oldest forms of actin, believed to have originated in unicellular organisms.



Fig 6-17 Fibroblast stress fibers visualized as bright linear structures stained with fluorescent antibodies to α -smooth-muscle actin. (Reprinted with permission from Giannopoulou and Cimasoni.⁴⁷ Original magnification $\times 900$.)

Actin is present in the cytoplasm in a dynamic equilibrium between its monomeric form and its polymeric or filamentous form. The monomeric (globular) form of actin assembles by noncovalent bonding to form narrow (7-nm) contractile microfilaments. Single molecules of actin are added and removed from the ends of the filaments in a treadmill fashion. The polymerization reaction requires adenosine triphosphate, magnesium, and a slightly acidic pH. The equilibrium between the two forms is regulated by capping proteins and filamentous actin-severing proteins. Gelsolin is a major actin-capping protein that increases the conversion of filamentous actin to globular actin in the presence of calcium. Actin and contractile systems are discussed further in chapter 11.

Fibroblast-to-matrix adhesion and traction

Fibroblasts attach to the substratum of the extracellular matrix via surface receptors for collagen and fibronectin. Attachment to the substratum is essential for cellular migration and for organization of the extracellular fibrillar matrix. The focal adhesion and its mature form, the fibronexus, have received a great deal of attention over the past decade.^{98,99}

In the formation of these adherent contacts, the cell membrane integrin α 5 β 1 attaches to the arginine-glycine-aspartic acid sequence of fibronectin (Figs 6-18 and 6-19).¹⁰⁰ Fibronectin molecules can polymerize to form pericellular matrices. Assembly is

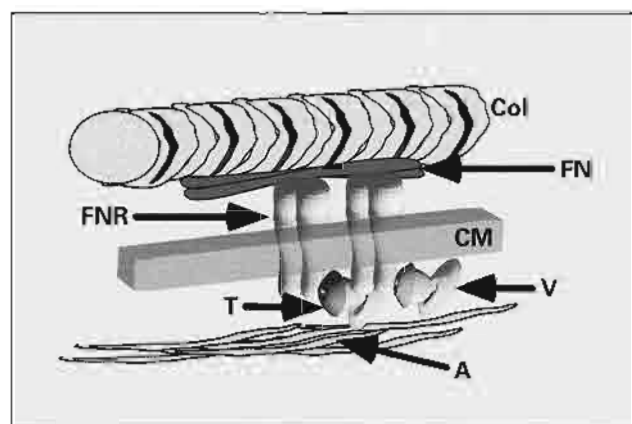


Fig 6-18 Arrangement of molecular components of the linkage between extracellular collagen fibrils (Col) and cytoplasmic actin filaments (A), mediated by fibronectin (FN), fibronectin receptor (FNR), talin (T), and vinculin (V). (CM) Cell membrane.

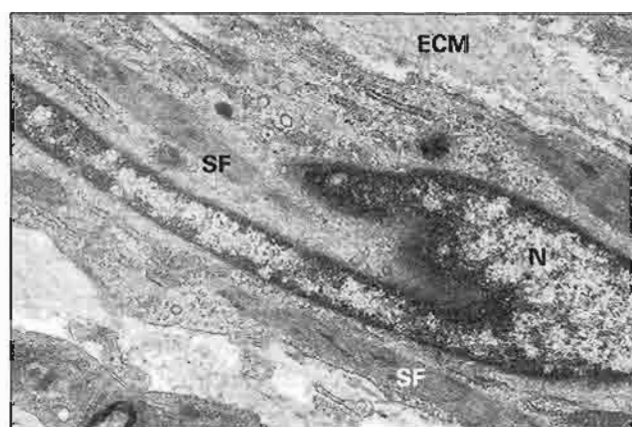


Fig 6-19a Periodontal ligament fibroblasts with stress fibers (SF). (ECM) Extracellular matrix; (N) nucleus. (Original magnification $\times 7,500$.)

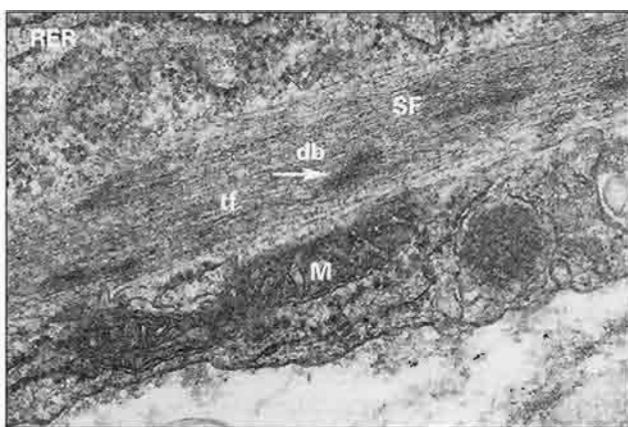


Fig 6-19b Stress fibers (SF) consisting of thin filaments (tf) and dense bodies (db). (M) Mitochondria; (RER) rough endoplasmic reticulum. (Original magnification $\times 31,000$.)

initiated by binding of soluble fibronectin molecules to the cell surface integrin receptors, $\alpha 5 \beta 1$ and $\alpha v \beta 3$.¹⁰¹ The cytoplasmic domain of the integrin receptor attaches to the peripheral cytoplasmic protein, talin, which in turn interacts with a protein called *vinculin*. Conformational changes in vinculin cause it to bind to actin microfilaments in the cortical cytoplasm, thereby completing a molecular bridge between the cell's contractile apparatus and fibronectin in the extracellular matrix.¹⁰²

With the binding of fibronectin to collagen fibrils, the molecular linkage extends from the cytoplasmic contractile apparatus to an extracellular collagen fiber network, establishing a mechanism for exerting traction on the collagen fibers (see Fig 6-18). In fibroblasts under tension, filamentous actin and smooth-muscle myosin associate to form contractile

stress fibers that terminate at the plasma membrane in fibronexus junctions (Figs 6-19 and 6-20). Through such cell-to-matrix contacts, the extracellular matrix can exert an effect on cell shape and behavior. Tension in the ECM is transmitted to fibroblast integrin receptors, leading to signaling events that alter the activity of the cell.

Human PDL fibroblasts respond to increased tension by upregulating the expression of IL-1 β ¹⁰³ and by secreting prostaglandin E₂.¹⁰⁴ In this "outside-in" type of signaling, tension transmitted to the fibroblast causes a rise in the activity of several small guanine triphosphatases, which regulate the enzyme cascades that lead to changes in cell shape and function. Cells can also alter the binding strength of their integrin receptors through cytoplasmic signaling pathways. This represents a form of "inside-out"

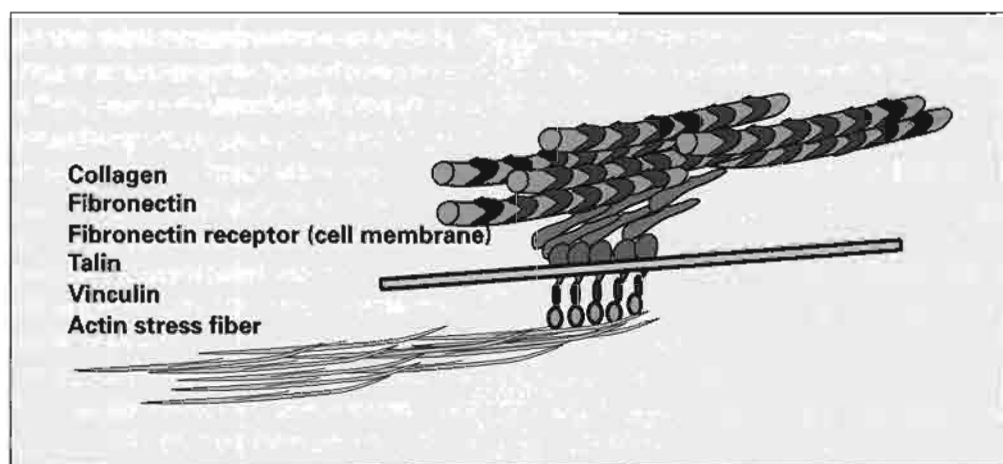


Fig 6-20 Fibronexus, composed of numerous assemblies of fibronectin and its receptor (see Fig 6-18), which form a colinear bond between extracellular collagen fibrils and a cytoplasmic bundle of actin filaments.

signal transduction.¹⁰⁵ The linkage between the cell surface and the immediate extracellular matrix serves as a node whereby the cell can receive regulatory information from the outside and provides a mechanism for exerting an organizing influence on the adjacent matrix.^{106,107}

When fibroblasts move through a collagen gel matrix, they tend to align collagen fibrils parallel to the long axis of migration.¹⁰⁸ If the substratum on which cells are placed is sufficiently anchored, traction exerted by the contraction of the actin network allows the cells to move over the substratum. When the substratum is not anchored, for example, the collagen fibrils in a collagen gel, the substratum (collagen fibrils) will be pulled toward the cells, and the collagen gel will be contracted into a tight ball of fibrils.⁴⁸

In vitro studies of matrix contraction have shown that fibroblasts are able to generate the same degree of tension that can be measured in a contracting wound.¹⁰⁹ Exposure of fibroblasts to cytochalasin D, a drug that causes disruption of actin networks, leads to a rapid decrease in the cell's ability to generate tension.¹⁰⁹ Platelet-derived growth factor (PDGF), insulin-like growth factor 1 (IGF-1), and TGF- β promote PDL fibroblast contraction of type I collagen gels.¹¹⁰ Platelet-derived growth factor promotes gel contraction by stimulating actin cytoskeletal polymerization and increasing the expression of integrins. Both gingival and PDL fibroblasts exhibit high gel-contracting abilities.¹¹¹ The contractile force gen-

erated by PDL fibroblasts has been measured to be approximately 2% that of smooth-muscle cells yet is sufficient to account for tooth eruption.^{48,112}

It has been estimated that each fibroblast may have a minimum of 105 fibronectin receptors displayed on its surface. On highly mobile fibroblasts, these receptors are diffusely distributed, while in stationary cells they are arranged in linear arrays, codistributed with cytoplasmic actin and extracellular fibronectin aggregates. Immunocytochemical localization of fibronectin in the periodontal ligament has shown that it forms aggregates about 90 nm thick on the surface of fibroblasts.¹¹³ These aggregates are usually codistributed with intracellular components of the fibronexus contact.²⁹

The fibronexus is a terminal for anchorage of stress fibers to the cell surface and extracellular matrix (see Figs 6-18 and 6-19). The stress fibers comprise well-defined bundles of actin and nonsarcomeric myosin oriented parallel to the long axis of the fibroblast (see Fig 6-19).⁴⁷ Stress fibers are found in fibroblasts involved in transferring tension to an extracellular fiber network that is firmly attached to stable structures in their immediate vicinity. Periodontal ligament fibroblasts can form robust stress fibers similar to those observed in myofibroblasts of healing wounds. Well-developed stress fibers and fibronexus contacts have been observed in fibroblasts of the transseptal fiber group between molar teeth of monkeys.⁴⁹

Fibroblast response to growth factors

The need to identify treatment modalities that can stimulate the wound-healing and regenerative abilities of periodontal tissues has sparked research into the response of PDL fibroblasts to various growth factors.¹¹⁴ Most of these studies have been conducted with cells grown in vitro.

Platelet-derived growth factor BB and IGF-1 have been found to have potent chemotactic and mitogenic effects on gingival and PDL fibroblasts.¹¹⁵⁻¹¹⁷ Both PDGF-BB and TGF- β stimulate collagen synthesis.

Transforming growth factor β also stabilizes collagen matrices by decreasing the synthesis and secretion of MMPs by fibroblasts. This effect is potentiated by increased secretion of plasminogen activator inhibitor 1, acting to decrease the conversion of plasminogen to plasmin. The main sources of PDGF and TGF- β in vivo are macrophages and platelets.

Periodontal ligament fibroblasts respond to parathyroid hormone in a characteristically osteoblastic way by increasing the synthesis of cyclic adenosine monophosphate.¹¹⁸

Basic fibroblast growth factor increases the proliferation of human PDL fibroblasts while it decreases the expression of alkaline phosphatase and the ability to form mineralized tissue.¹¹⁹

Epidermal growth factor and its receptor appear to maintain the PDL fibroblast phenotype. Differentiation of cementoblasts and osteoblasts involves a downregulation of the receptor for epidermal growth factor.¹²⁰

Periodontal ligament fibroblasts cultured in vitro produce a peptide that has been shown to have a chemotactic effect with specificity toward PDL fibroblasts.¹²¹ Because gingival fibroblasts do not respond to this protein, there is hope that clinical use of this peptide may become possible in reattachment procedures where recruitment of PDL fibroblasts is needed.

Clinical Correlations

Regeneration and repair of the periodontal ligament

The PDL, like other connective tissues, has a high potential for regeneration and repair.¹²²⁻¹²⁵ Regeneration of a functional ligament requires correlated development of new cementum and bone for the attachment of Sharpey's fibers. Furthermore, the re-

generation of these tissues must be regulated to prevent fusion of bone to cementum (ankylosis).

Repair of the PDL involves the replacement of small areas of damaged ligament. This process can be considered similar to the connective tissue component of normal wound healing in other tissues. In repair, new fibroblasts are derived from perivascular progenitor cells in the adjacent normal PDL. Migration of fibroblasts into the area to be repaired is facilitated by the presence of fibrin and fibronectin networks. New collagen fibers are laid down rapidly and often without functional orientation or attachment to the adjacent hard tissues. Reorganization of the initial collagen matrix into oriented principal fiber bundles requires continued cellular activity over several weeks.

Studies of potential progenitor-cell pools have shown that the marrow spaces of the alveolar bone, particularly along lateral communications between the PDL and the marrow, are sites of cell proliferation. Of interest is the finding that newly divided cells from these sites appear to contribute to new cementum formation as well as to the deposition of new PDL collagen. Although there is still uncertainty surrounding the origin of cementoblasts and osteoblasts in the PDL, evidence suggests that cells of the osteoblastic subtype develop from perivascular cells in the PDL proper as well as from the progenitor cells arising from adjacent marrow compartments. After extensive damage to the PDL connective tissue, the PDL compartment is populated by an increased number of bone-forming cells, and ankylosis of the tooth to the alveolar bone usually results.

Surgical attempts to regenerate new PDL attachment have revealed that success depends on the following principles:

1. After removal of inflamed tissue, the root surface must be debrided of contaminants, such as bacterial endotoxins. Endotoxins adhering to the hydroxyapatite crystals of cementum interfere with cell attachment.
2. Gingival epithelial cells must be prevented from gaining access to the root surface. Conditions that favor growth of PDL fibroblasts over gingival fibroblasts must be created. This is accomplished by the use of resorbable membranes to exclude gingival tissues from making contact with the root surface during PDL regeneration.^{125,126}
3. The geometric nature of the lesion to be repaired is a factor in the prognosis for success. Defects with intact lateral bone surfaces and with an

ample amount of normal PDL adjacent to the area to be repaired are more likely to undergo satisfactory regeneration than are lesions that have horizontal loss of attachment.

Morphologic and functional restoration of the periodontium is unpredictable, and often the best that can be attained is a wound-healing reaction rather than regeneration. In a wound-healing reaction, fringe fiber layers are only rarely produced with functional attachment to the old collagen of the root. Presumably the right combination and the most appropriate stimulatory substances have yet to be identified. The search for growth factors and attachment molecules that will permit successful and predictable regeneration procedures is actively being pursued in numerous laboratories and clinical research centers.

The application of growth factors during periodontal therapy has increased the chances for attaining complete regeneration. Recently it was discovered that a single application of PDGF and IGF to the root surface produced new cementum with functionally oriented PDL fibers and new crestal alveolar bone 4 weeks postsurgery in monkeys.¹²⁷ The level of regeneration appears significantly greater when IGF is added to PDGF than when either growth factor is used alone. Histologic study of the sites of new attachment revealed new cementum and well-developed Sharpey's fiber bundles.¹²⁸ This report is particularly interesting in view of the fact that the combination of PDGF and IGF, among several growth factors tested in vitro, is the most potent stimulator of PDL fibroblast chemotaxis, proliferation, and collagen synthesis.¹²⁹ Regeneration of the PDL is accelerated by the application of PDGF-BB to acid-demineralized root surfaces in membrane-guided repair of furcation defects in beagle dogs.

Osteogenic protein 1, also known as *bone morphogenetic protein 7*, a member of the TGF- β family of growth factors, has produced significant new bone, cementum, and periodontal ligament within 8 weeks following its application to furcation defects in beagle dogs.¹²⁸

Bone morphogenetic protein 2, a growth factor that induces osteoblast differentiation from undifferentiated connective tissue cells, has also proven to be a promising agent for the clinical induction of new bone formation during surgical repair of alveolar bone defects.¹³⁰

The implication of amelogenin in the early stage of cementoblast differentiation (see chapter 7) has led to clinical experimentation and eventual application

of a commercial amelogenin preparation to promote new cementum formation and reattachment of PDL fibers.¹³¹⁻¹³³ Recent studies have shown that amelogenin acts as a cell adhesion factor.¹³⁴

Doxycycline suppression of MMPs in the treatment of periodontitis

The discovery of the anticollagenase action of doxycycline has led to its use as an adjunct therapeutic agent in the treatment of periodontal disease and other diseases that have tissue breakdown as an element of their pathogenesis.¹³⁵ Doxycycline acts through its ability to bind Ca^{++} and Zn^{++} , both cations essential for MMP enzyme activity.¹³⁶ Of additional significance is the fact that doxycycline decreases cytokine, nitric oxide, and prostaglandin production.¹³⁷ Furthermore, doxycycline appears to increase the anabolic functions of connective tissue cells. Clinical trials of the administration of a low-dose formulation of doxycycline in the treatment of periodontitis have shown lower levels of MMP activity in sulcular fluid and a significant reduction in the loss of connective tissue attachment.^{135,138}

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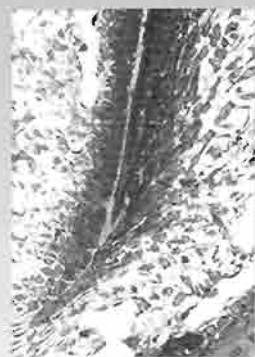
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Chapter 7

Root Formation and Cementogenesis



During the past decade, research in regeneration of the periodontal attachment received new impetus from the discovery of connective tissue growth factors and cell adhesion molecules. The opportunity to create conditions for successful periodontal regeneration has been improved by the use of biocompatible and biodegradable membranes to exclude inappropriate cell types from colonizing the root surface during postsurgical wound healing. Furthermore, the application of growth factors to stimulate cell differentiation and the application of conditioning agents to increase the adhesiveness of the root surface have achieved promising results. The key to obtaining successful reattachment to a previously diseased root surface is the formation of new extrinsic fiber cementum with embedded Sharpey's fibers. Clinicians and dental researchers are now focusing greater attention on cementum in the hope of discovering methods for inducing its formation.

Schroeder's book on the periodontium¹ should be consulted for a comprehensive review of the classic histologic and ultrastructural aspects of cementum. The light and electron microscopic micrographs that illustrate that work are of outstanding quality and highly instructive. The newest molecular aspects of cementum composition and formation have been reviewed, with a focus on tissue repair, by Saygin and colleagues.² In addition, Diekwisch has contributed new findings to support the classic view of the mesenchymal nature of cementogenic cells.³

Development of the Roots

Ooe has described the early morphogenetic events of root formation in human teeth in a remarkably well-illustrated book, *Human Tooth and Dental Arch Development*.⁴ He described the three-dimensional growth of human teeth using scale models constructed meticulously from serial histologic sections.

At the cap stage of human tooth development, the apical rim of the enamel organ forms the edge of the apical foramen, a space through which the dental papilla maintains continuity with the dental sac and adjacent mesenchyme. The circumferential apical rim does not lie on a single plane, because the vestibular (labial) and lingual portions extend deeper into the jaw. As the tooth bud continues to grow, the apical foramen enlarges and the vestibular and lingual extensions of the enamel organ give rise to epithelial interradicular processes. The interradicular processes lengthen by expansion of the enamel organ, rather than by cell proliferation within the processes. The distances between the tips of the interradicular processes remain nearly constant during this phase of coronal expansion. These events have been illustrated by Ooe⁴ in reconstructed models of the human primary second molar at various stages of development (Fig 7-1).

In trifurcated maxillary molars, a third epithelial interradicular process originates from the distal apical rim of the enamel organ. Thus, the distal part of the

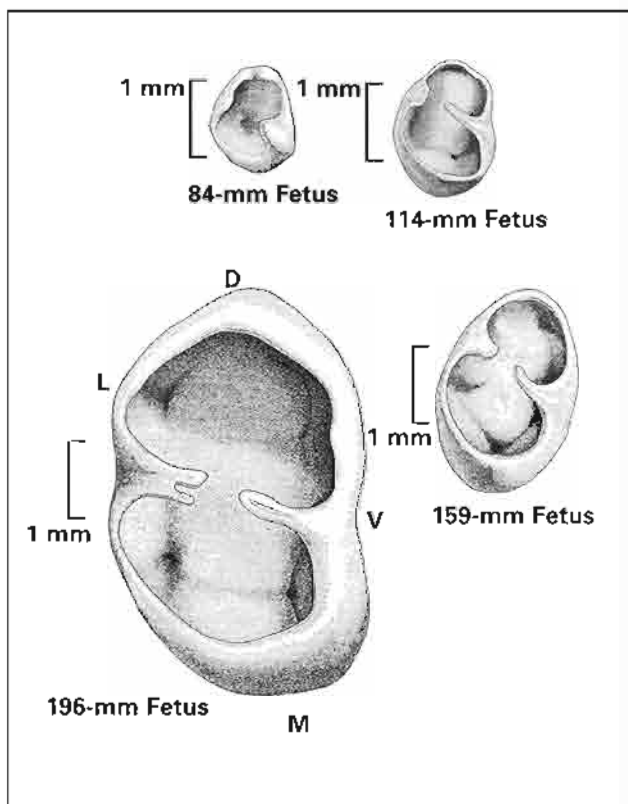


Fig 7-1 Models of the mandibular primary second molar reconstructed from serial sections at four stages of fetal development. All tooth buds are drawn to scale and are viewed in the apical-to-coronal direction. (D) Distal; (V) vestibular; (M) mesial, (L) labial. (Adapted with permission from Ooe.⁴)

original apical foramen becomes divided by a third process into a distolingual and a distomesial root primordium. The epithelium of the interradicular process retains its odontogenic potential and is responsible for inducing the differentiation of odontoblasts. Fusion of the epithelial interradicular processes and induction of dentinogenesis leads to the formation of the dentinal floor of the pulp chamber. Small deposits of enamel, produced by ameloblast differentiation of the epithelial cells of the interradicular processes, may be formed on the external surface of the floor of the pulp chamber.

It is not uncommon for the epithelial interradicular processes to split, forming islands of epithelial tissue prior to dentinogenesis. If the epithelial islands fail to fuse prior to formation of dentin and cementum, small communications are created between the pulp

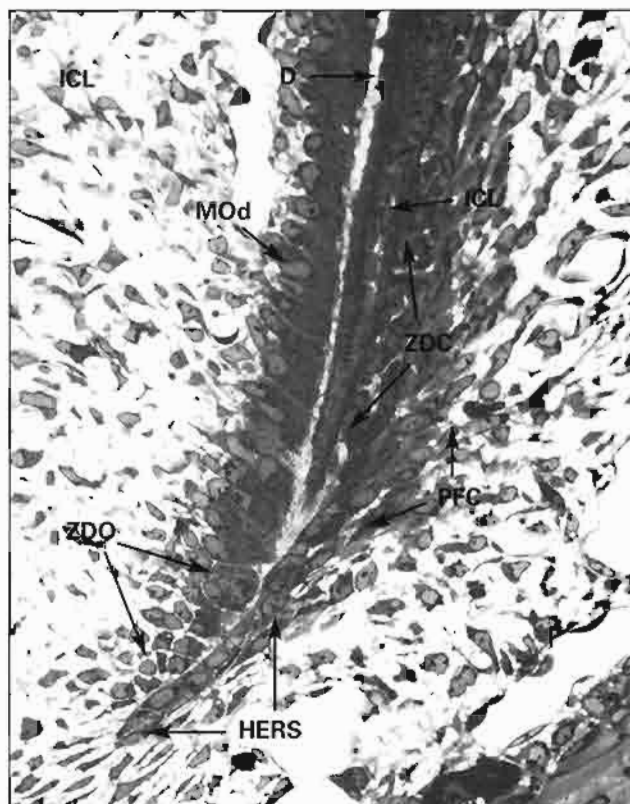


Fig 7-2 Histologic section of the leading edge of root formation. Hertwig's epithelial root sheath (HERS) initiates odontoblast differentiation and subsequently undergoes disintegration, thereby exposing the newly formed dentin matrix to perifollicular cells (PFC). The PFCs move to the dentin surface and initiate the formation of acellular extrinsic fiber cementum (D) Dentin; (ICL) initial cementum layer; (MOD) mature odontoblasts; (ZDC) zone of developing cementum; (ZDO) zone of differentiating odontoblasts. (Toluidine blue stain. Original magnification $\times 320$.)

chamber and the coronal part of the interradicular periodontal ligament (PDL). These may persist in the fully formed tooth, to become pathways of infection between the pulp and the PDL.

Hertwig's epithelial root sheath (HERS), a double layer of epithelial cells, is continuous with, and extends apically from, the apical rim of the enamel organ. At this stage, the sheath forms a circumferential band between the pulpal ectomesenchyme and the follicular and perifollicular ectomesenchyme (Fig 7-2). Apical growth of HERS occurs by proliferation of the epithelial cells of the sheath. Continuity between the enamel organ and HERS is lost soon after root formation begins.

The apical region of the developing root contains ectomesenchymal progenitor cells that give rise to fibroblasts, preodontoblasts, and precementoblasts.

The coordinated proliferation of the epithelial and ectomesenchymal cells at the apical site gives rise to cells needed for elongation of the root and formation of mineralized tissue.

Preodontoblasts differentiate adjacent to the inner layer of the root sheath and its basal lamina (see Fig 7-2). The inner layer of the root sheath appears to perform the same inductive functions attributed to the inner enamel epithelium during coronal odontoblast development. Slavkin and colleagues have reported that HERS secretes polypeptides related to enamelin and amelogenin proteins.^{5,6} In contrast, others have failed to find amelogenin protein at the developing root surface.³

The potential inductive effects of enamel matrix proteins on pulpal preodontoblasts and on perifollicular fibroblasts and precementoblasts have not been established. However, an epithelial-mesenchymal interaction between cultured cells of HERS and fibroblasts has been demonstrated *in vitro*.⁷ When fibroblasts were grown with cells of HERS, the fibroblasts showed increases in rough endoplasmic reticulum, Golgi membranes, and associated secretory granules as well as increases in secretion of collagen.

The epithelial root sheath persists over the root surface for a short time after the odontoblasts begin secreting dentin.^{8,9} At that time, the root sheath undergoes partial disintegration as epithelial cells separate and become displaced away from the newly formed dentin matrix.⁸ Some epithelial cells survive in the PDL to form the epithelial rests of Malassez (Fig 7-3).^{1,10,11} Other epithelial cells may undergo programmed cell death or transdifferentiate into mesenchymal cells. On disintegration of the root sheath, the follicular ectomesenchymal cells begin to form cementum when directly exposed to the newly deposited dentin and/or matrix products previously secreted by HERS.

Follicular cells adjacent to the cervical part of the crown may deposit spurs of acellular afibrillar cementum (AAC) over the cervical enamel.^{1,12} Formation of AAC occurs when the reduced enamel epithelium detaches from the enamel surface. The exposed enamel appears to stimulate secretion of the matrix of AAC by follicular connective tissue cells. A scanning electron microscopic study of the cemento-enamel junction of human teeth revealed that overlap of AAC over enamel was a common finding; the highest incidence (about 50%) was in molars.¹³ At the electron microscopic level, the matrix of AAC consists of a fine granular substance rich in glycosaminoglycans and a nonfibrillar collagenous component.¹ The functional significance of AAC is unknown.

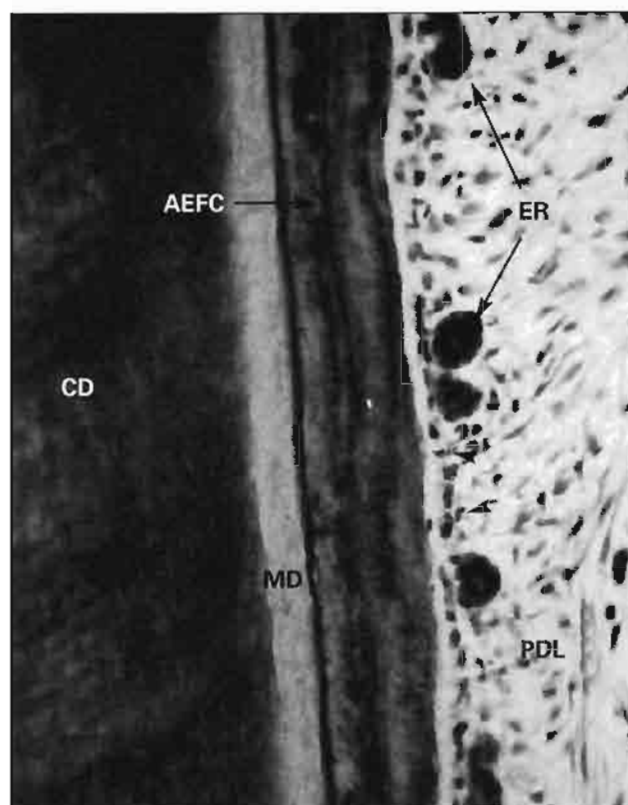


Fig 7-3 Histologic section depicting acellular extrinsic fiber cementum (AEFC) covering the mantle dentin (MD). Remnants of the root sheath persists as epithelial rests (ER) in the periodontal ligament (PDL). Note the layer of cells (arrowheads) along the surface of the AEFC. (CD) Circumpulpal dentin. (Hematoxylin-eosin stain. Original magnification $\times 220$.)

Deposition of root cementum begins just apical to the cervical enamel. Root cementum exists in several histologic types¹: acellular extrinsic fiber cementum (AEFC), acellular intrinsic fiber cementum (AIFC), cellular intrinsic fiber cementum (CIFC), and cellular mixed stratified cementum (CMSC).^{1,11,14,15}

Acellular extrinsic fiber cementum lacks cells and is composed of densely packed striated collagen fiber bundles embedded in a granular matrix rich in glycosaminoglycans. The fibers are oriented perpendicular to the root surface (Sharpey's fibers) and continuous with the principal fibers of the PDL. Acellular extrinsic fiber cementum is the only form of cementum on the coronal part of roots, covering from 40% to 70% of the root surface. It is present as a thin

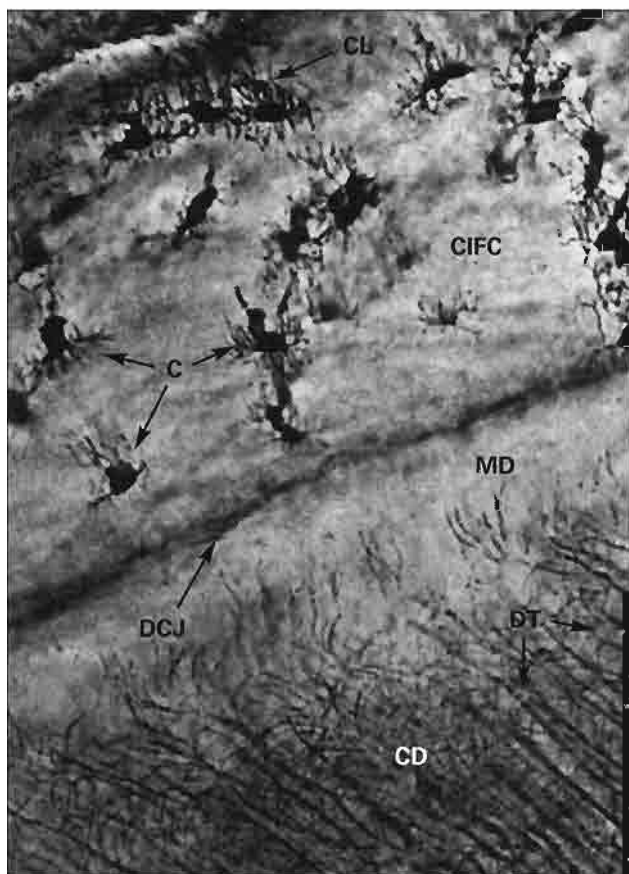


Fig 7-4 Histologic section of root surface depicting cellular intrinsic fiber cementum (CIFIC) containing numerous cementocytes (C). Note the radiation of canaliculi from the cementocyte lacunae (CL) toward the periodontal ligament space. (CD) Circumpulpal dentin; (DCJ) dentinoceamental junction; (DT) dentinal tubules; (MD) mantle dentin. (Hematoxylin-eosin stain. Original magnification $\times 400$.)

layer, 20 to 250 μm deep, usually containing several incremental lines (see Fig 7-3).¹ It serves the exclusive function of anchoring the root to the PDL. The rate of appositional growth of AEFC is extremely slow, less than 0.1 μm per day.

Cellular mixed stratified cementum, ranging in thickness from 100 to 600 μm , is made up of alternating layers of AEFC and CIFIC/AIFC. It is located primarily on the apical third of the root and in the furcation area of multirooted teeth. Cellular mixed stratified cementum serves to reshape root surfaces to accommodate for physiologic drift and nonphysiologic shifting of teeth in the tooth socket and for the repair of resorption sites.^{1,15} Cellular mixed stratified cementum is covered by a thin layer of AEFC for attachment to the PDL.¹

Cellular intrinsic fiber cementum contains cementocytes embedded in a collagenous matrix of intrinsic collagen fibers (Fig 7-4). Scanning electron micrographs of human root surfaces have clearly demonstrated that intrinsic fibers lie mostly parallel to the root surface and run a circular or spiral course around the root.¹ Sharpey's fibers do not penetrate into the CIFIC. In addition, CIFIC is found in old resorption lacunae and in root fracture planes. Its function is associated with repair and adaptation. The rate of formation of CIFIC is much more rapid than that of AEFC, ranging from 0.5 to 3.0 μm per day, about equal to the rate of dentin formation in developing teeth, but slightly slower than the rate of bone deposition.

Acellular intrinsic fiber cementum is formed by cementoblasts secreting in a unipolar mode.¹⁶ By secreting matrix slowly from one surface, the cementoblasts avoid subsequent entrapment in matrix as cementocytes.

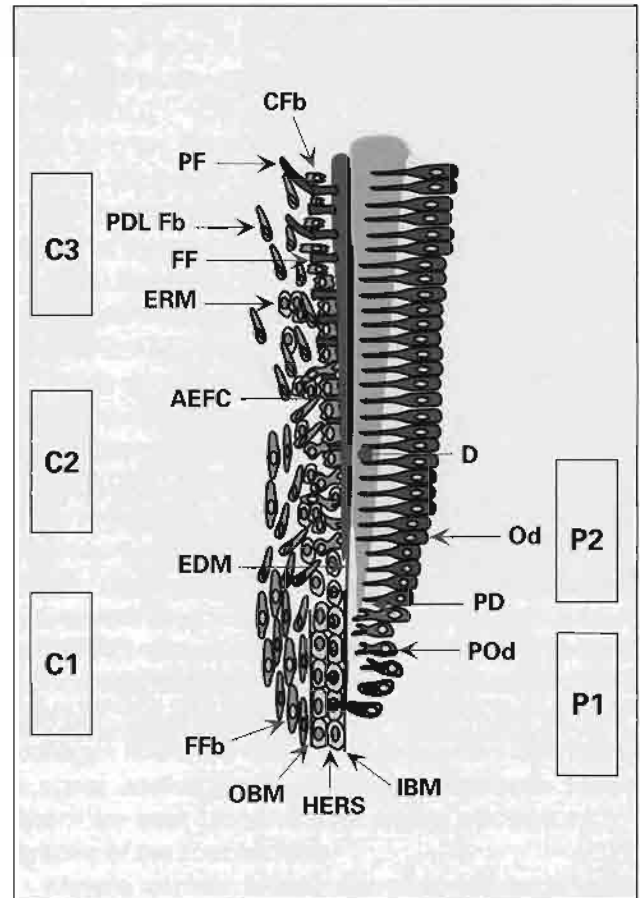
Cementogenesis in Animal Models

Most research on cementum has been carried out in experimental animals (dogs, rats, and mice), frequently as a component of studies of the periodontal ligament or of root development.¹⁷⁻²⁰ Such studies have described the development, biologic potential, microanatomy, and physiologic responsiveness of cementum in animals. Not all of the knowledge gained from such studies is applicable to humans.^{1,14,15} Based on what has been learned from other organ systems, however, it is reasonable to expect that there is a significant carryover. Apparent differences may become resolved as the molecular events of cementogenesis are better understood.

The role of HERS in root development, especially relating to the initiation of cementogenesis, has become a focus of considerable attention.²¹ Because the epithelial cells of the inner layer of HERS are analogous to the preameloblasts, it was suggested early on that they might secrete enamel matrix proteins over the newly deposited root dentin.^{5,7,17,18} Based on various studies, it is now generally accepted that there is a transient period of secretion of proteins, including bone sialoprotein (BSP), osteopontin (OPN), and amelin, by the cells of HERS²²⁻²⁴ (Fig 7-5).

In addition to these matrix proteins, components of the epithelial basement membrane, such as laminin and collagen type IV, are included in the narrow band of matrix juxtaposed to the dentin matrix.

Fig 7-5 Development of acellular extrinsic fiber cementum (AEFC), from the early induction (C1), to differentiation and secretion (C2), to anchorage to the periodontal ligament (PDL) via the merger of fringe fibers (FF) to the principal fibers (PF) of the PDL (C3). Preodontoblast (POd) differentiation (P1) and dentin (D) secretion (P2) occur at slightly earlier time frames. Ectomesenchymal cells of the immature dental pulp become polarized toward Hertwig's epithelial root sheath (HERS) in the early development of the preodontoblasts (P1). Cementum formation occurs after the induction of preodontoblasts. During C1 and P1, the epithelial root sheath is intact. It is bordered by an inner basement membrane (IBM) facing the pulp and an outer basement membrane (OBM) facing the fibroblasts of the dental follicle (FFb). The FFb lie parallel to the long axis of the HERS and appear relatively undifferentiated while in the C1 zone. During C1, the cells of HERS deposit a thin layer of organic matrix (epithelial cell-derived matrix, EDM) against the newly secreted predentin. Transition between C1 and C2 is marked by disappearance of the basal laminae, separation of the epithelial cells, and polarization of FFb toward the root surface. New collagen fibers, secreted by the polarized FFb, intermingle with the nonmineralized fibers of the predentin matrix (PD). During C2 and early C3, the FFb at the root surface hypertrophy and take on a cuboidal shape. At this stage, the cells have a cementoblastic phenotype. The newly secreted collagen fibers are bundled in intercellular compartments to form fringe fibers (FF). These extrinsic fibers eventually merge with developing principal fibers (PF) of the PDL. Following the early formation of FFs (C3), the cementogenic cells at the root surface appear to become less active and to take on a fibroblast-like morphology (CFb). (ERM) Epithelial rest of Malassez; (Od) odontoblast; (PDL-Fb) periodontal ligament fibroblast.



This layer is sometimes identified as *intermediate cementum*, a misleading term because the matrix in question is a product of epithelial and dentinogenic cells.¹⁶ According to Schroeder¹ and Bosshardt and Selvig,¹⁵ no such layer is interposed between cementum and dentin in human teeth.

The potential role of these epithelial matrix molecules in triggering the differentiation of cells capable of forming AEFC and CFC is a primary question that remains mostly unanswered. Nevertheless, the concept that epithelial (enamel organ) proteins stimulate cementogenesis has found clinical application in experimental tissue regeneration protocols. It has been reported that the application of hydrophobic amelogenin peptides to denuded root surfaces promotes new cementum formation.²⁵

The fate of HERS following the onset of cementogenesis is also a subject of unresolved debate. Traditional thinking proposed that HERS disintegrated into small clusters and/or strands of epithelial cells that

survived indefinitely in the periodontal ligament. More recent studies have suggested that epithelial cells might undergo epithelial-mesenchymal transition into fibroblasts and cementoblasts that deposit acellular and cellular cementum, respectively.²¹ The possibility that some epithelial cells of the root sheath undergo epithelial-mesenchymal transformation and subsequently secrete cementum matrix must be investigated further. There is evidence that cells of the inner layer of the root sheath become incorporated in cellular cementum or trapped between cementum and dentin during formation of the apical part of the root.^{26,27} However, the evidence that many of the cells of the root sheath retain an epithelial phenotype, and survive in the PDL as the epithelial rests of Malassez, is incontrovertible (see Figs 7-3 and 7-5).¹

In developing rat molar roots, formation of AEFC occurs only after HERS is invaded by cells of the adjacent follicular ectomesenchyme (see Figs 7-2 and 7-5).^{8,16} These polarized cells extend cytoplasmic

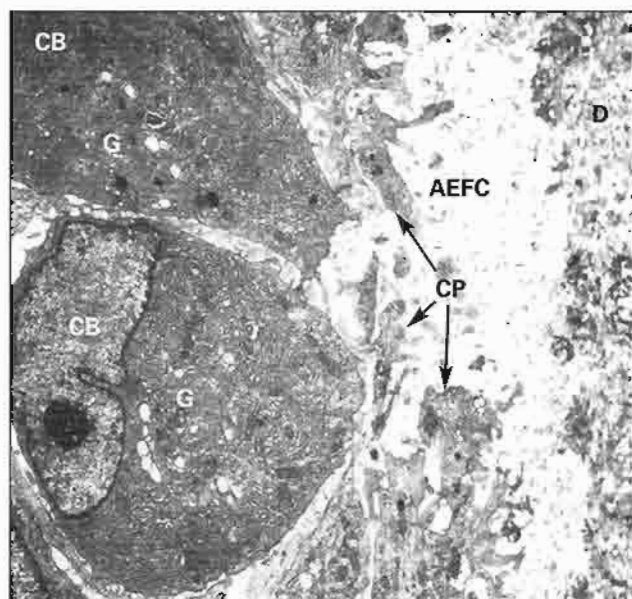


Fig 7-6 Large polarized cementoblasts (CB) on the root surface during initial acellular extrinsic fiber cementum (AEFC) formation in the mouse molar. Note the well-developed Golgi complex (G) and the many cell processes (CP) adjacent to the zone of matrix secretion. (D) Dentin. (Original magnification $\times 5,300$.)

processes between the epithelial cells of the root sheath toward the dentin surface (Figs 7-5 and 7-6).⁸ The follicular cells appear to migrate to the dentin surface concomitant with the breakup of the root sheath. These morphologic changes suggest that during formation of AEFC the cells of the dental follicle respond to a chemoattractant present in the dentin matrix or to one produced by the inner epithelial layer of the root sheath. Spreading and hypertrophy of the follicular cells follow contact with the dentin surface (see Figs 7-5 and 7-6).

Immunohistochemical studies have shown that the cells that line the developing cementum contain BSP, OPN, and osteocalcin, proteins typically found in osteoblasts and bone matrix.^{28,29} The localization of BSP and OPN at the site of the initial mineralization of AEFC suggests that precementoblasts and/or fibroblasts of the follicular connective tissue bind to arginine-glycine-aspartic acid sequences of BSP and OPN via cell surface integrins during cementogenesis.²⁸⁻³⁰ The OPN component also appears to serve as an adhesive factor to bind the newly secreted collagen fibrils to the root surface. When AEFC production is reactivated during wound healing, a dense, granular, OPN-rich nonfibrillar matrix is deposited as

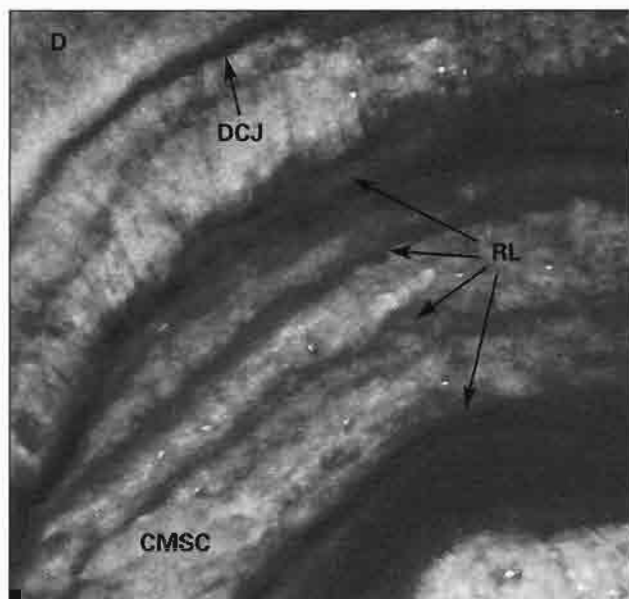


Fig 7-7 Histologic section of cementum in the furcation between roots. Cellular mixed stratified cementum (CMSC) contains several reversal or "cement" lines (RL). CMSC fulfills adaptive function between the dentinocemental junction (DCJ) and the periodontal ligament. (D) Dentin. (Hematoxylin-eosin stain. Original magnification $\times 425$.)

a thin layer between the old and the new AEFC. These thin layers are visualized in histologic sections as densely stained reversal or "cement" lines (Fig 7-7).

The cells responsible for depositing the first layer of AEFC exhibit a high level of basophilia, consistent with a well-developed rough endoplasmic reticulum (see Fig 7-6).³¹ These cells are also characterized by a high level of alkaline phosphatase.³² Specific collagen secretory granules are formed in a large and conspicuous Golgi complex (see Fig 7-6). The secretory activity of the AEFC matrix has been documented with electron microscopic autoradiography in which tritiated mannose was used as an indicator of glycoprotein synthesis during AEFC formation in rat molars.³¹ Following a brief initial phase of rapid AEFC formation, the cementum-forming cells lose their cuboidal shape and appear to join the spindle-shaped cells of the PDL (see Fig 7-5).⁸ The relationship between the shape of cementoblasts and the orientation of collagen fibers has been analyzed extensively (see "Basic Science Correlation: Construction of the Attachment," later in this chapter).

Recent studies of the deposition and mineralization of AEFC in rats have shown that fibroblast alkaline phosphatase is a driving force for the mineral-

ization of the matrix.³² Alkaline phosphatase, in the presence of a source of organic phosphate, increases the amount of phosphate bound to collagen fibrils and thereby increases the rate of mineralization of the fibrils.

Cellular cementum begins to form when the tooth comes into occlusion. The formation of this bonelike tissue involves differentiation of precementoblasts derived from the developing PDL. Proliferation of the root sheath ceases at the same time that cellular cementum is formed. Epithelial cells may become entrapped in the matrix during formation of cellular cementum.

Cementogenesis in Humans

During human tooth development, HERS does not remain in contact with the root surface following odontoblast differentiation.^{11,33} Hertwig's epithelial root sheath detaches from the dentin surface very close to the apical edge of the developing root. After the detachment and disintegration of HERS, AEFC forms at the growing root tip when fibroblasts of the dental follicle make contact with the unmineralized surface of dentin matrix. According to Bosshardt and Schroeder, fibroblasts secreting in a unipolar direction deposit and bundle collagen fibrils at the dentin surface to form a thin layer of perpendicularly oriented "fringe fibers."³³ The collagen fibrils of the fringe fibers appear to interdigitate and thereby become linked with the unmineralized dentin collagen fibers at the dentinocemental junction. The AEFC-forming cells have sheathlike cytoplasmic processes that delineate extracellular compartments, within which the fringe fibers are assembled.^{33,34} Formation of AEFC proceeds lengthwise along the developing root at a rate of about 5 to 7 μm per day, requiring 43 to 65 months for completion in human premolars.¹¹

As the dentin mineralization front advances to reach the outermost part of the mantle dentin, it contacts the fringe fibers and they undergo slow mineralization to complete the process of AEFC formation. The first evidence of mineralization in the fringe fibers appears in the central core of each fiber bundle, presumably by epitaxy from the mineralized dentin.³³ With time, the mineralization spreads across the entire width of the fringe fibers, and the resulting uniform mineralization front subsequently advances in proportion to the growth of the AEFC. Whether or not the AEFC-forming fibroblasts deposit special glycoproteins and/or glycosaminoglycans needed for the supramolecular organization of collagen fibers, or for the support of mineralization, re-

mains to be established. After the fringe fibers reach a length of about 20 μm , they become associated and continuous with the principal fibers developing in the PDL.¹¹

During the life of the tooth, the AEFC continues to grow in thickness at a slow rate of 1.5 to 3.0 μm per year. Closely spaced incremental (cement) lines suggest that the growth of AEFC is episodic. Presumably the PDL cells adjacent to the root surface respond to appropriate environmental signals calling for an increase in AEFC matrix and its mineralization.

When root development is about two thirds completed and the tooth is about to enter its functional stage, cementum formation converts from AEFC to a CMSC (CIFC/AIFC) type.^{15,33} The conditions and factors responsible for this transition are unknown. The formation of CIFC closely resembles formation of bone. Cementoblasts and cementocytes are involved in the secretion of intrinsic fibers (in contrast to the PDL fibroblasts that produce extrinsic fibers).

The rate of apposition of CMSC (about 0.1 to 0.5 μm per day) is less than that of bone.³⁵ The intrinsic collagen fibers are assembled in bundles that follow a spiral course along and around the root. These fibers are best observed in scanning electron micrographs of the root surface.¹

Mature cementoblasts are relatively large cells with a highly basophilic cytoplasm. During CIFC formation, they secrete in a relatively rapid multipolar mode and become entrapped in the matrix as cementocytes.^{1,11,16,36,37} Slow matrix deposition is thought to occur in a unipolar fashion during AIFC formation, permitting the cementoblasts to escape entombment in the matrix. Cementoblasts share similar morphologic features with osteoblasts, suggesting that these two cell types might originate from a common progenitor pool located in the PDL and the marrow spaces of the adjacent alveolar bone.

Responsiveness of Cementum

Examination of histologic sections of human teeth, particularly teeth that have a history of periodontal disease and/or traumatic occlusion, reveals the presence of reversal lines (cement lines). These lines provide evidence that cementum on the root surface is far from an inactive or unresponsive tissue.³⁰ When cementum (and bone) stops forming, a resting line is deposited.

Resting lines stain intensely with hematoxylin and metachromatic dyes, indicating increased amounts of glycosaminoglycans and/or glycoproteins (see

Fig 7-7).³⁸ Immunohistochemical methods have been used to detect osteopontin in reversal lines.³⁰ The study of reversal lines can provide clues to sites of prior resorption, repair, and the cessation and activation of both AEFC and CFC deposition. In general, reversal lines increase in number with age, reflecting the growth and resorption history of the tooth.^{39,40}

Cementum is more resistant to osteoclastic resorption than is bone. Because of this difference, dentists are able to move teeth through bone by exerting light pressure on teeth. Excessive pressure on the PDL leads to cellular damage and a resulting inflammatory response and root resorption. Cementoclasts (essentially osteoclasts that resorb cementum) have the same morphology as osteoclasts. The properties of cementum that give it greater resistance to resorption probably do not reside in its matrix (which is essentially similar to bone). The greater resistance may be due to the inaccessibility of the mineralized cementum surface, which is covered by tightly packed, nonmineralized collagen fibrils (see Fig 7-3). It is well established that osteoclastic differentiation is aided by the chemoattractant properties of osteocalcin and by contact of the preosteoclasts with a mineralized surface (see chapter 8). The observation that unmineralized cementum of rat molar teeth resists resorption during distal drift illustrates the point that an unmineralized collagenous surface affords a degree of protection against osteoclasts.⁴¹

Cementoblasts express parathyroid hormone receptors, but unlike osteoblasts and bone-lining cells, they do not retract in response to parathyroid hormone to expose the root surface to preosteoclasts.⁴² Differential responses of cementoblasts to parathyroid hormone, as well as to other factors that have parathyroid hormone-like effects, might protect the root from osteoclastic attack by reducing the opportunity for attachment and differentiation of cementoclasts.

Another difference between bone and cementum is the high fluoride content of cementum.⁴³ The elevated fluoride content of cementum may contribute to its greater resistance to resorption.

Localized damage in the PDL, or at the root surface, leads to localized root resorption that may include the removal of dentin. Root resorption is often a consequence of acute dental trauma and the use of excessive force during orthodontic tooth movement. Areas of resorption are found along the compressed PDL and root surface.⁴⁴

The first step in root resorption is the degradation of the collagenous matrix by fibroblast and mono-

cyte metalloproteinases.⁴⁵ These enzymes are activated during the inflammatory response related to the removal of necrotic tissue. This leads to exposure of the mineralized cementum surface and the release of factors that stimulate the differentiation and attachment of osteoclasts. A viable bone marrow adjacent to the site of injury creates a more vigorous osteoclastic response, presumably because it is a source of cementoclast precursors.

Root resorption is followed by a repair phase during which new cementum (CFC and/or CMSC) is deposited in the resorption defect.⁴⁶ Attachment is provided by the initial deposit of AEFC over the old surface.⁴⁶ Mononuclear cells invade the defect from normal root surfaces bordering the defect. These cells lay down a thin layer of AEFC in contact with the old cementum and/or dentin surface.¹⁵ Over a 6- to 8-week period, the bulk of the resorption cavity is filled with CFC.⁴⁷ Bone morphogenetic protein 7, released from the cementum and dentin during resorption, may function, as it does in bone, as a coupling factor to attract cementogenic cells to the root surface. With time a new layer of AEFC forms over the CFC to reestablish a PDL attachment.

New collagen fibrils and the old collagen fibrils are connected either by direct splicing of the new collagen to the ends of the old collagen fibrils or by an intermingling of old and new fibrils. Several investigators have observed that surface demineralization occurs during the natural healing process, indicating that root preparation with demineralizing agents during a surgical reattachment procedure is an unnecessary step. Removal of hydroxyapatite crystallites during the naturally occurring repair process exposes the ends of the old collagen fibrils to newly secreted procollagen molecules. Chances that a functional PDL attachment will occur requires that epithelial cells be prevented from making contact and attaching to the root surface.

Rat cementoblasts and their precursors express growth hormone receptors.⁴⁸ Growth hormone receptor is expressed in precementoblasts adjacent to HERS. Receptor expression increases during cementum formation and thereafter declines in cementocytes. Periodontal ligament cells next to AEFC do not express growth hormone receptor. Excessive amounts of growth hormone cause hypercementosis.⁴⁹ In contrast, hypophysectomy leads to reduced amounts of cellular cementum. In humans with growth hormone deficiency, some teeth fail to form and others undergo delayed eruption.

Matrix Proteins, Adhesion Molecules, and Growth Factors of Cementum

Cementum matrix is made up predominantly of type I collagen and glycosaminoglycans.⁵⁰ Chondroitin sulfate is the most abundant glycosaminoglycan in cementum. Smaller amounts of dermatan sulfate and hyaluronic acid are also present.^{51,52} Chondroitin sulfate is concentrated in the perilacunar matrix surrounding cementocytes.⁵³

The bone matrix proteins, osteocalcin, osteopontin, osteonectin, and bone sialoprotein, are expressed by cementoblasts and deposited in cellular cementum.^{28,54–58} Several research groups have extracted attachment proteins from cementum. Somerman and colleagues extracted osteopontin from mature human and bovine cementum and the extracellular matrix adjacent to the epithelial root sheath.⁵⁹ They demonstrated that it enhances the attachment of dental ectomesenchymal cells to culture dishes. Bone sialoprotein has been identified in bovine cementum matrix.⁵⁹ Immunohistochemical studies have demonstrated bone sialoprotein on the surface of alveolar bone and AEFC at the time of mineralization.²⁹

Osteopontin has been localized at the electron microscopic level in dense planar deposits at cement lines. Osteopontin mediates cell attachment and the cohesion of matrix molecules at incremental lines. McKee et al³⁰ have suggested that OPN, based on its localization and known bonding action, is the first and last product to be secreted by osteoblasts and cementoblasts.

Bone sialoprotein and osteopontin contain mineral-binding domains and arginine-glycine-aspartic acid sequences that mediate cell attachment to mineralized tissue. Of interest is the observation that endotoxin blocks cell attachment by interfering with the cell-binding sites of the cemental sialoprotein.

Cementum matrix also contains an attachment protein that does not cross-react with other known attachment proteins such as osteopontin and bone sialoprotein. This cementum-specific attachment protein binds to the hydroxyapatite component of cementum.^{60,61} It stimulates chemotaxis of bone cells and fibroblasts and increases the attachment of these cells to the cementum.^{62,63}

In addition, a cementum growth factor has been isolated from cementum.^{64–66} It acts as a mitogen for fibroblasts and vascular smooth-muscle cells. Bone morphogenetic protein 7, also known as *osteogenic*

protein 1, a well-known bone induction factor, has been demonstrated to induce new cementum on surgically denuded root surfaces in the baboon.⁶⁷

A study of the localization of various adhesion molecules and integrins in the PDL has shown that tenascin (cytotactin) is concentrated near the surface of cementum and bone, while fibronectin is distributed more evenly across the ligament. Although the significance of adhesion molecules in the behavior of PDL cells has yet to be established in vivo, it is reasonable to expect that they regulate cellular activity in the microenvironment of the root surface.

Process of Tooth Eruption

Several studies over the past three decades have implicated the migration and traction of periodontal fibroblasts as the motive force behind tooth eruption.^{68,69} In the developing periodontal ligament, the apical zone has the highest rate of fibroblast proliferation. According to the PDL traction theory of tooth eruption, it is the migration of daughter cells coronally, away from the proliferation compartment, that pulls the tooth toward the oral mucosal surface. Traction exerted by the fibroblast contractile apparatus on the extracellular collagen matrix via cell-to-matrix focal contacts must be transmitted to the tooth through the insertion of periodontal fibers into the cementum.

The observation that thermal injury to the PDL in the rat incisor slows the eruptive process is taken as evidence for a role of PDL fibroblasts in tooth eruption. Colchicine, a substance that blocks cell division by interfering with the microtubules of the mitotic spindle, has been shown to decrease eruption in a dose-dependent manner.^{70–72} Colchicine also blocks cell migration by interfering with the transport of new membrane and protein to the cell surface as well as the secretion of new collagen from the leading edge of the cell.⁷³ However, the observation that the PDL does not have functionally oriented principal fibers during the rapid phase of eruption is problematic for the fibroblast traction theory.¹⁰

Other investigators have focused attention on vascular-tissue hydrostatic pressure within the periodontal ligament as a contributor to eruptive force.^{74–76} The “water-binding” properties of proteoglycans, which contribute to osmotic pressure in the PDL, are key components of this theory. This hemodynamic hypothesis is also supported by experiments with vasodilating drugs⁷⁷ and by evidence that sympathectomy causes a significant acceleration of incisor eruption in the rat.⁷⁸

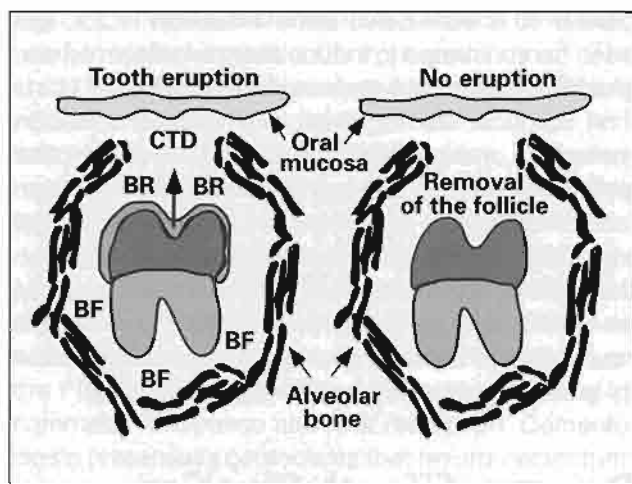


Fig 7-8 Influence of the tooth follicle (green) on bone resorption (BR) along the eruption pathway. (BF) Bone formation; (CTD) connective tissue domain.

In recent years, dental scientists have come to realize that tooth eruption is a complex process not easily explained by a single mechanism, whether it be cell proliferation, fibroblast traction, or vascular-tissue pressure. The focus is now on biologic mediators of bone and connective tissue remodeling as key factors in tooth eruption. Recent advances were summarized in two conferences on the biologic mechanisms of tooth eruption.^{79,80}

Several biologic regulators of tooth eruption were identified in a series of research studies spanning the last two decades (reviewed by Wise⁸¹ and Marks⁸²). The eruption of teeth requires bone resorption to enlarge a pathway through the alveolar bone (Fig 7-8).^{83,84} A narrow band of connective tissue penetrates the alveolar bone to connect the developing tooth to the sub-mucosal connective tissue. During eruption this connective tissue canal is widened by osteoclastic bone resorption. Additionally, new bone formation at the base of the bony crypt creates an outward eruption force directed against the base of the erupting tooth.

Morphologic studies of experimental surgical interventions have provided evidence that the postsecretory enamel organ and the highly vascularized dental follicle connective tissue coordinate the erup-

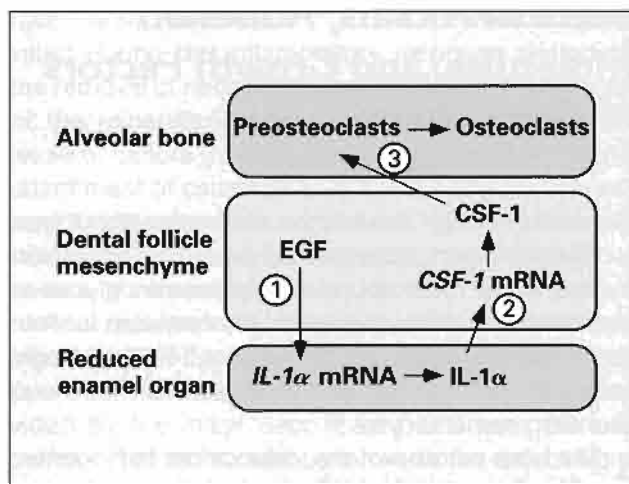


Fig 7-9 Proposed molecular signals originating in the dental follicle mesenchyme and adjacent epithelial cells of the reduced enamel organ that regulate bone resorption during tooth eruption. Epidermal growth factor (EGF) stimulates the production of interleukin 1 α (IL-1 α) in the epithelium (1). In turn, IL-1 stimulates the production of colony-stimulating factor 1 (CSF-1) and monocyte chemoattractant protein 1 (2), which act to attract mononuclear preosteoclasts to the adjacent alveolar bone (3). Osteoclastic bone resorption widens a path through the alveolus to permit eruption. (Based on reports from Marks⁸² and Wise.⁸¹)

tion of teeth.^{81,85} The presence of the dental follicle was found to be essential for bone resorption during the formation of the eruptive pathway as well as for new bone formation apical to the erupting tooth (see Fig 7-8).^{86,87} Supporters of the concept that the dental follicle regulates the eruption of teeth point to the fact that proteinase activity in the follicular connective tissue peaks at the initiation of tooth eruption.⁸⁸ The observation that rootless teeth undergo eruption⁸⁵ is further convincing proof for the integrated activity of bone resorption and bone formation under the control of the dental follicle during eruption. Furthermore, when bone resorption is blocked with bafilomycin A, a drug that interferes with vacuolar adenosine triphosphatase, tooth eruption stops.⁸⁹ In osteopetrosis, a condition wherein bone resorption is defective on a systemic level, tooth eruption either fails or is severely retarded.⁹⁰

Monocytes containing tartrate-resistant acid phosphatase, an indicator of lysosomal activity, invade the connective tissue of the dental follicle early in tooth development and during tooth eruption.^{91,92} These cells are believed to be osteoclast precursors. Osteoclasts containing tartrate-resistant acid phosphatase are present in large numbers on adjacent

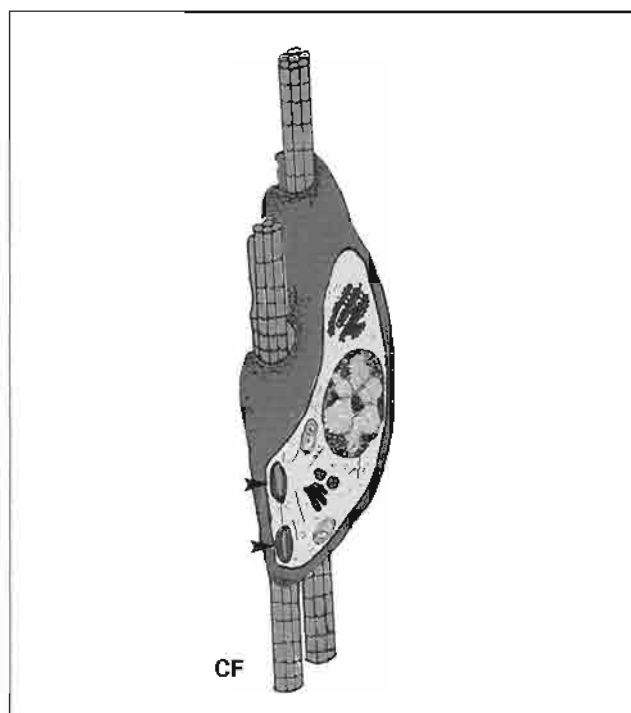


Fig 7-10 Perifollicular cell located adjacent to the outer layer of the root sheath. (In Figs 7-10 to 7-13, the root surface is at the left of the cell.) The cell is oriented parallel to the root sheath and large collagen fiber bundles (CF). Note the alignment of secretory granules (arrowheads) to collagen bundles. (Adapted from Yamamoto et al³⁴ with permission from Springer-Verlag.)

bone surfaces of erupting teeth. Monocyte chemoattractant protein 1, originating in the dental follicle, acts as a chemoattractant for these cell types.⁹³

Recent evidence shows that colony-stimulating factor 1 and epidermal growth factor are involved in tooth eruption.⁹⁴⁻⁹⁷ Isolated cells of the dental follicle secrete colony-stimulating factor 1, a substance involved in the recruitment and differentiation of pre-osteoclasts. Epidermal growth factor upregulates the production of colony-stimulating factor 1 via its ability to stimulate the cells of the reduced enamel organ to make interleukin 1 α (Fig 7-9).^{61,98,99}

Basic Science Correlation: Construction of the Attachment

The attachment of the principal fibers of the PDL to the root surface provides an informative example of the role that cells play in organizing and orienting extracellular fibers into functional networks. Scanning

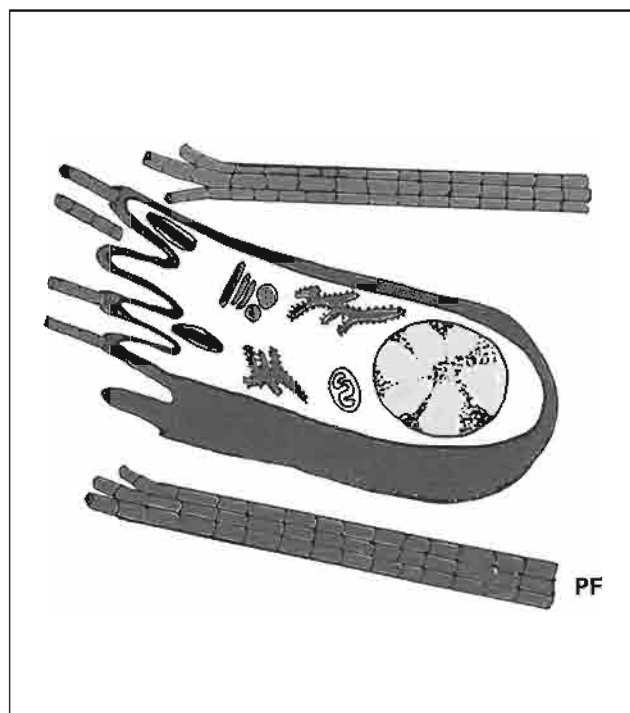


Fig 7-11 Perifollicular cell advancing toward the dentin surface after the disintegration of the root sheath. Large peripheral fiber (PF) bundles are now positioned in the lateral intercellular spaces. They became established as fringe fibers. Numerous cell processes extend from the anterior end of the cell toward the dentin. Small collagen fibers are secreted from these cytoplasmic processes. These fibers are the first components of the initial layer of cementum. (Adapted from Yamamoto et al³⁴ with permission from Springer-Verlag.)

electron microscopy has been helpful in establishing the three-dimensional shape of the perifollicular cells (PFCs), cementoblasts, and fibroblasts of the PDL. Using both scanning electron microscopy and conventional transmission electron microscopy, investigators have determined the shape of the cells that make cementum.

Prior to the onset of cementogenesis, the PFCs nearest to the root sheath are aligned parallel to the epithelial cells.^{8,34} Collagen bundles that lie parallel to the root sheath are partly enveloped in cytoplasmic grooves formed by the PFCs (Fig 7-10). Cytoplasmic microtubules and collagen secretory granules are oriented in the same direction as the extracellular collagen fibers.

With the onset of the disruption of the root sheath, the PFCs assume an elongated profile with polarity toward the dentin matrix (Fig 7-11). The cells appear to move toward the dentin in the spaces created by the disruption of the root sheath. During shifting of the PFCs, the collagen bundles that were initially par-

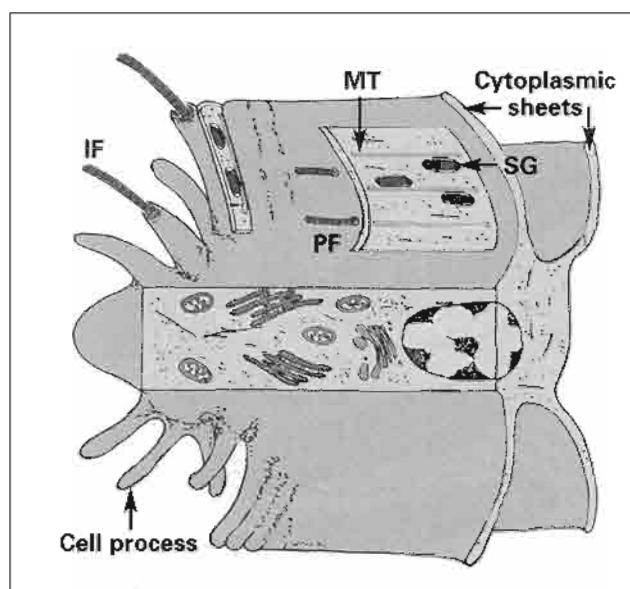


Fig 7-12 Cell involved in the dual activity of enlarging the circumference of a fringe fiber (Sharpey's fiber) and adding new intrinsic fibers (IF) to the cementum. Note the thin cytoplasmic sheets that partially encircle the fringe fibers and the alignment of secretory granules (SG) and microtubules (MT) in the cytoplasm. At the anterior end of the cell, the lateral sheets are replaced by small cytoplasmic processes. Intrinsic fibers are secreted from the process in various orientations. (PF) Peripheral fiber. (Adapted from Yamamoto et al³⁴ with permission from Springer-Verlag.)

allel to the root sheath are reorganized, so that they come to lie in the lateral intercellular spaces between the PFCs, oriented perpendicular to the root surface (see Fig 7-11).^{8,34}

Many small cytoplasmic processes extend from the leading edge of the PFCs. Collagen fibrils secreted from these leading-edge processes intermingle with the collagen of the dentin matrix. Although many of the small collagen fibers appear to have no preferred orientation, most are aligned perpendicular to the root by the microtubule-secretory granule apparatus in the cytoplasmic processes (see Fig 7-11).³⁴

In a later stage of AEFC formation, fibroblasts (or PFCs) extend thin cytoplasmic sheets that partially surround the developing fringe fibers, or Sharpey's fibers (Fig 7-12). These sheets or veils of cytoplasm are best developed on the part of the cell nearest the PDL. Examination of the cytoskeleton in the cytoplasmic sheets reveals that the microtubules and collagen secretory granules are aligned mostly parallel to the fringe fibers, indicating that fringe fibers grow in circumference by secretion from the surface of cy-

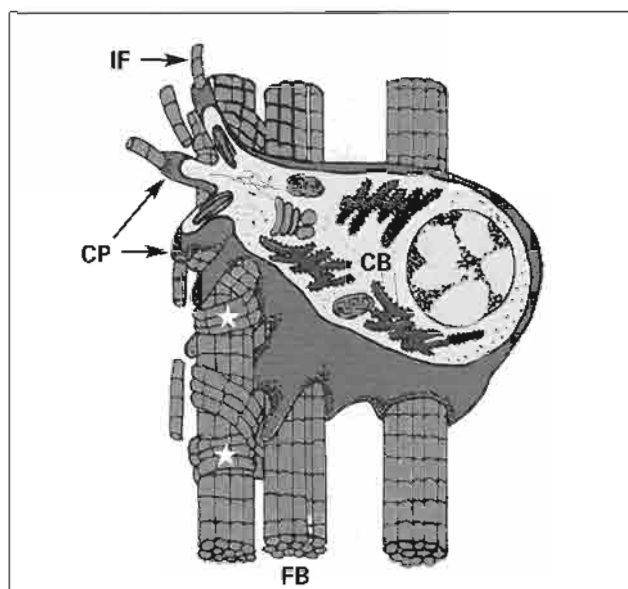


Fig 7-13 Cementoblast (CB) engaged in the deposition of cellular intrinsic fiber cementum. Large collagen fiber bundles (FB) are laid down parallel to the root surface. Smaller intrinsic fibers (IF), deposited from cell processes (CP) at the anterior portion of the cell, wrap around the larger fiber bundles (stars). Transmission electron microscopy has revealed parallel alignment of microtubules and collagen secretory granules in the cell processes with the extracellular collagen fibrils. (Adapted from Yamamoto et al³⁴ with permission from Springer-Verlag.)

toplasmic sheets.³⁴ In contrast, small cytoplasmic processes that give rise to intrinsic fibers (see Fig 7-12) characterize the end of the cell near the dentin (or the previously deposited cementum).

In the early development of cellular intrinsic fiber cementum, cementoblasts appear to deposit fiber bundles parallel to the surface of the root. Subsequently, the cementoblasts engage in binding these fibers with smaller intrinsic fibers deposited from cytoplasmic processes extending from the end of the cell facing the dentin (Fig 7-13).^{34,36} Transmission electron microscopic analysis of the small cell processes shows that microtubules align collagen secretory granules parallel to the developing intrinsic fibers. In the formation of CIFC, these cementoblasts are eventually surrounded by matrix as new waves of cementoblasts differentiate at the cemental surface.

Although the full story has yet to be developed, preliminary evidence suggests that cells orient newly deposited collagen by aligning secretory granules parallel to microtubules within the cyto-

plasmic processes and sheets that demarcate extracellular microcompartments.^{73,100} Cell migration and/or movements of cell processes, controlled by the cytoplasmic actin contractile network, could also play a role in organizing the fibers of the extracellular matrix. This has been observed in developing tendons as well as in the PDL. The cell surface receptors and cytoplasmic signaling steps that control the flow of cell membrane components and secretory granules to the cell surface, and the subsequent cell-matrix interactions needed to construct the fibrous architecture of a specific tissue, are undoubtedly complex.

Clinical Correlation: Cementum Hypoplasia

Cementum hypoplasia occurs on root surfaces of patients who suffer from hypophosphatasia.^{101,102} Hypophosphatasia is a hereditary disease transmitted as an autosomal-recessive trait. Cementum formation on the primary anterior teeth is usually defective. Premature loss of teeth without root resorption is one of the first signs of the disease. Other skeletal abnormalities are also present.¹⁰² Severely affected individuals do not survive beyond childhood.

In patients with localized juvenile periodontitis, the permanent incisors and first molars show advanced pocket formation, loss of attachment, and advanced alveolar bone resorption. In the familial form of localized juvenile periodontitis, tissue destruction develops rapidly without an associated inflammatory process. Root surfaces of the affected teeth have hypoplastic cementum.^{103,104} In some of these patients, serum alkaline phosphatase is abnormally low (hypophosphatasia). A decreased efficiency of the neutrophil response to periodontal pathogens has also been reported in patients with localized juvenile periodontitis.

The pathogenesis of tooth loss in these two diseases underscores the importance of normal levels of alkaline phosphatase during periods of root development. Low levels of alkaline phosphatase lead to defects in formation and mineralization of cementum. Root surfaces devoid of normal AEFC and/or CMSC have a deficient attachment of collagen fibers and are thus more susceptible to the consequences of bacterial colonization of the root surface. Under these conditions, rapid loss of attachment, concomitant with epithelial migration and alveolar bone resorption, leads to premature tooth loss.

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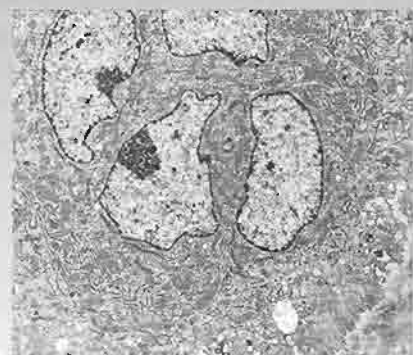
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Chapter 8

Bone



Bone is a remarkably strong biologic construction material. It has a tensile strength comparable to that of cast iron, and it has a breaking stress point in bending that is intermediate between those of hard wood and cast iron, despite the fact that it is only about a third as heavy as iron. These properties are attributable to the engineering principles of hollow tubular construction, lamination, and internally reinforced matrix. The properties of bone are all the more remarkable because it is a dynamic tissue, undergoing constant renewal in response to mechanical, nutritional, and hormonal influences.

Bone provides a protective covering for the vulnerable brain, spinal cord, and thoracic viscera, rigid internal supports for the extremities and the articulations, and attachments for muscles necessary for locomotion. In addition, bone functions as a reservoir of calcium that can be drawn on to meet unusual metabolic needs not satisfied by dietary intake.

The scientific and medical literature that constitutes the body of knowledge relating to bone is enormous. For in-depth reviews of bone biology, the reader should consult recent monographs.¹⁻⁵

Types and Functions of Osteogenic Cells

The osteogenic line of cells consists of preosteoblasts, osteoblasts, osteocytes, and bone-lining

cells. Osteogenic cells arise from primitive mesenchymal cells contained in the stroma of bone marrow and from pericytes adjacent to small blood vessels in connective tissue.⁶⁻⁸ Differentiation of osteogenic stem cells requires stimulation by transforming growth factor β (TGF- β), and bone morphogenetic protein 2 (BMP-2).⁶ Differentiation markers include the expression of osteocalcin, osteonectin, alkaline phosphatase, and bone sialoprotein.

Preosteoblasts

Periosteal and connective tissue preosteoblasts have the morphologic appearance of an inactive fibroblast, containing many free ribosomes, only a few profiles of rough endoplasmic reticulum (RER), and a small Golgi complex. During differentiation, preosteoblasts make contact with adjacent preosteoblasts or with previously differentiated osteoblasts, develop cytoplasmic polarity, and greatly increase the amount of RER and Golgi cisternae.

Mesenchymal cell differentiation into the osteogenic cell line is preceded by the activation of the *Osf2/Cbfa1* gene, which appears to serve as a master gene to turn on the expression of osteocalcin, osteopontin, bone sialoprotein, and collagen synthesis.^{9,10} The *Osf2/Cbfa1* protein is induced by bone morphogenetic protein 7 and is decreased by vitamin D₃.⁹ Experimental studies have detected high levels of *Osf2/Cbfa1* expression in the developing

mandible and maxilla as well as in tooth buds.^{9,11} Mutations in *Osf2/Cbfa1* block bone matrix formation and cause cleidocranial dysplasia, an autosomal-dominant disease characterized by a variety of skeletal abnormalities, including short stature, poorly developed clavicles, and supernumerary teeth.¹²

Osteoblasts

Osteoblasts secrete the collagenous and noncollagenous proteins and the proteoglycans of bone matrix. Osteoblasts also secrete matrix metalloproteinases (MMPs) into the extracellular bone matrix in an inactive form, along with tissue inhibitors of metalloproteinases.^{13,14} Regulatory cytokines and growth factors are also important secretory products of mature osteoblasts. Among these factors are regulators of osteoclast development and the differentiation of various hematopoietic cell lines.

Alkaline phosphatase is expressed at high levels in osteoblasts and is preferentially distributed along the apical surface and on cytoplasmic processes extending into the osteoid layer.¹⁵ The enzyme is anchored to the external surface of the plasma membrane by linkage to phosphatidylinositol.¹⁶ Alkaline phosphatase is also released in soluble and insoluble forms by osteoblasts.¹⁷ The level of alkaline phosphatase in serum is a systemic indicator of bone formation.

Osteoblasts also contain plasma membrane calcium adenosine triphosphatase (ATPase), also known as the *calcium pump*, a transporter that actively pumps Ca^{++} into the extracellular space using the energy of adenosine triphosphate (ATP) hydrolysis.¹⁸ Although it has been suggested that the calcium pump is involved in mineralization, its preferential localization to the distal surface (facing connective tissue) of the osteoblasts suggests that it may be involved in mobilizing calcium from bone rather than in concentrating Ca^{++} for mineralization.¹⁹

To meet their high level of protein synthesis and secretion, osteoblasts contain an abundance of RER and large Golgi complexes (Fig 8-1). In routine hematoxylin-eosin-stained sections viewed in a light microscope, the osteoblast is intensely basophilic and its Golgi complex appears as a pale-staining region situated between the nucleus and the apex of the cell adjacent to the bone surface. On rapidly forming bone surfaces, osteoblasts range in height from cuboidal to low columnar and are tightly packed in a pseudoepithelial fashion along the bone (Fig 8-2). Osteoblasts express a specific cadherin (OB cadherin) during differentiation.²⁰ Close, side-by-side

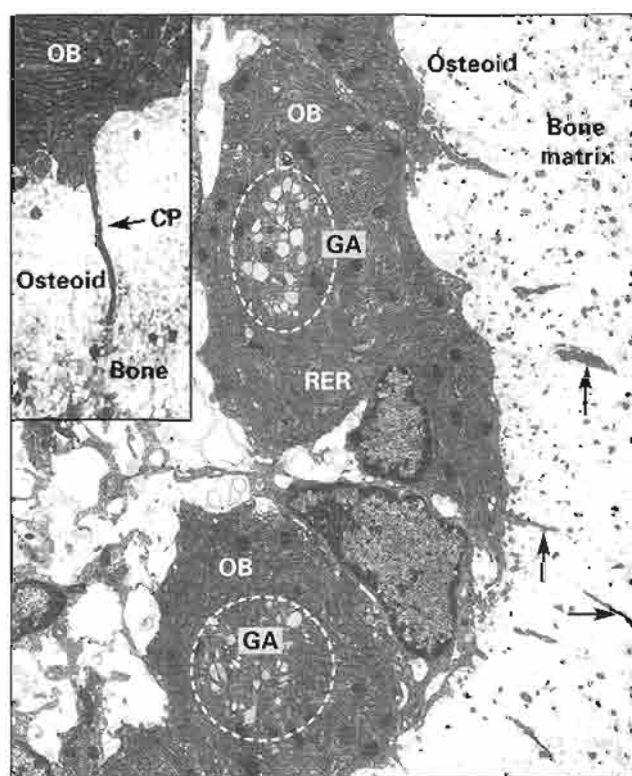


Fig 8-1 Osteoblasts (OB) forming intramembranous bone exhibit well-developed rough endoplasmic reticulum (RER) and Golgi apparatuses (GA). Osteoblast cell processes (arrows) in longitudinal and cross section penetrate into the osteoid and bone matrix. (Original magnification $\times 3,600$.) Inset: Higher magnification of osteoblast cell process (CP). (Original magnification $\times 6,000$.)

contact and Ca^{++} -dependent adhesion are characteristic features of active osteoblastic cells.

Immunocytochemical studies of the localization of connexins in sites of early mandibular bone formation indicate that gap junctions develop between condensing mesenchymal preosteoblasts just prior to production of osteoid matrix and remain between mature osteoblasts and osteocytes in fully developed bone.²¹⁻²³

Electron microscopy provides additional evidence that adjacent osteoblasts form gap junctions and adhesive contacts across narrow intercellular spaces. During mineralization of the bone matrix, the lateral intercellular spaces appear to be sealed by tight junctions, thereby creating a bone compartment distinct from the general interstitial spaces.²⁴ All osteoblasts have a secretory apical surface in contact with bone. Sometimes the osteoblast is oriented at an angle to the bone surface and the secretory pole

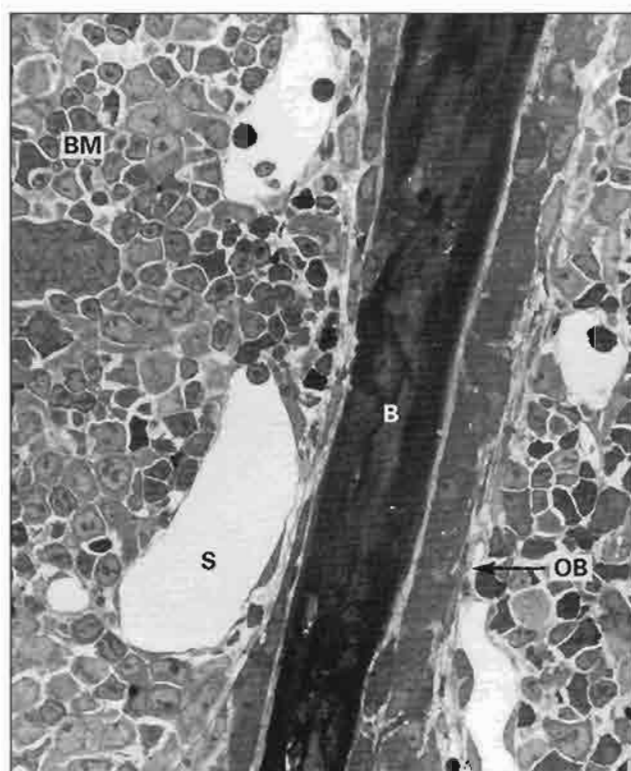


Fig 8-2 Endosteal bone trabecula (B) illustrating active surface covered by intensely basophilic osteoblasts (OB). (BM) Bone marrow cells; (S) sinusoidal space. (Original magnification $\times 280$.)

takes the form of a tapering cytoplasmic process lying parallel to the osteoid.

Ninety percent of bone matrix consists of type I collagen (with a minor fraction of type V collagen). The remaining 10% of bone matrix is composed of several noncollagenous proteins and small proteoglycans (decorin and biglycan). The osteoblast's Golgi complexes, and the presecretory and secretory granules that arise in it, resemble those observed in active fibroblasts and odontoblasts. Secretory granules, roughly 300 nm long and about 30 nm wide and containing a moderately dense filamentous material, are present in the Golgi complex and in the apical cytoplasm. It is unclear if all secretory products (collagen, noncollagenous protein, and proteoglycan) are packaged together or if separate secretory granules are formed for each of the secretory products.

An intact microtubular network is required for the translocation of secretory granules into the secretory

pole of the cell. Fusion of the granules to the cell membrane and the extrusion of their contents give rise to unmineralized bone matrix, or the osteoid layer. Osteoid, like predentin, must undergo a period of "maturation" before it becomes mineralized. Thus, there is a band of osteoid approximately 10 μm deep between osteoblasts and the mineralization front. Numerous cytoplasmic processes arising from the apical cell surface of the osteoblast penetrate the osteoid layer. These cytoplasmic processes make gap junctional contact with cytoplasmic processes arising from osteocytes.

Communicating networks of osteoblastic cells have been most extensively studied in cell culture. Parathyroid hormone (PTH), prostaglandin E_2 (PGE_2), and TGF- β increase gap junction coupling between osteoblastic cells.²⁵⁻²⁷ Gene transcription of bone matrix proteins, osteocalcin, and bone sialoprotein is modulated by gap junction communication.²⁸ Loss of intercellular communication leads to a decline in the cyclic adenosine monophosphate (cAMP) response to PTH.²⁹

Adhesion of osteoblasts to the underlying bone surface is mediated by plasma membrane integrins.³⁰ Rat tibial osteoblasts express $\alpha 5\beta 1$, $\alpha v\beta 3$, $\alpha 3\beta 1$, $\alpha 6\beta 1$, and $\alpha 1\beta 1$ integrin heterodimers that are located in plasma membrane attachment plaques.³¹ The binding of the $\beta 1$ integrin subunits to extracellular ligands (collagen and/or fibronectin) is essential for normal bone formation.³² Fibronectin, an early secretory product of differentiating osteoblasts, appears responsible for the initial attachment of osteogenic cells at the extracellular site of bone formation. Factors that decrease the expression of $\beta 1$ subunits, such as glucocorticoids, disrupt the organization of the osteoblastic layer and diminish the formation of osteocytes. Integrins also mediate the attachment of osteoblasts to metallic implant materials.³³ Experimental evidence indicates that attachment to components of the extracellular matrix mediates signaling cascades through activation of focal adhesion kinase.³⁴

In addition to their primary function, which is the production of bone matrix, osteoblasts also express growth factors, chemokines, and prostaglandins that act in an autocrine fashion to regulate osteoblastic activity and in a paracrine manner to influence other cells, especially osteoclasts. Transforming growth factor β , a secretory product of the osteoblast, acts as an autocrine factor to stimulate osteoblastic activity. Transforming growth factor β and its receptors have been localized in bone cells and matrix in areas of intramembranous and endochondral bone forma-

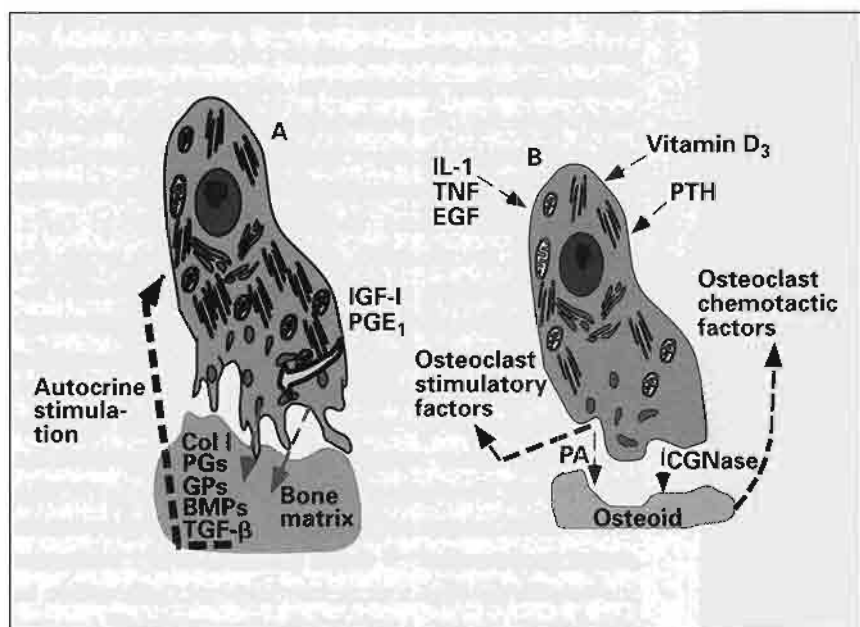


Fig 8-3 Osteoblast (A) in the bone matrix secretory mode and (B) in the retraction or deactivation mode. Paracrine factors, such as insulin-like growth factor I (IGF-I) and prostaglandin E₁ (PGE₁), stimulate matrix production, consisting of collagen type I (Col I), proteoglycans (PGs), glycoproteins (GPs), bone morphogenetic proteins (BMPs), and transforming growth factor β (TGF- β). Transforming growth factor β also acts in an autocrine fashion to stimulate secretion. Downregulation of osteoblastic activity is effected by interleukin 1 (IL-1), tumor necrosis factor (TNF), and epidermal growth factor (EGF). These cytokines also cause osteoblasts and/or preosteoblastic stromal cells to release substances that are essential for osteoclast development, such as monocyte colony-stimulating factor 1 and osteoclast differentiation factor/tumor necrosis factor superfamily 11. Parathyroid hormone (PTH) and 1,25-dihydroxy-vitamin D₃ cause osteoblast retraction and the secretion of collagenase (CGNase) and plasminogen activator (PA). Degradation of the osteoid matrix releases factors (osteocalcin) that are chemotactic for osteoclast precursors.

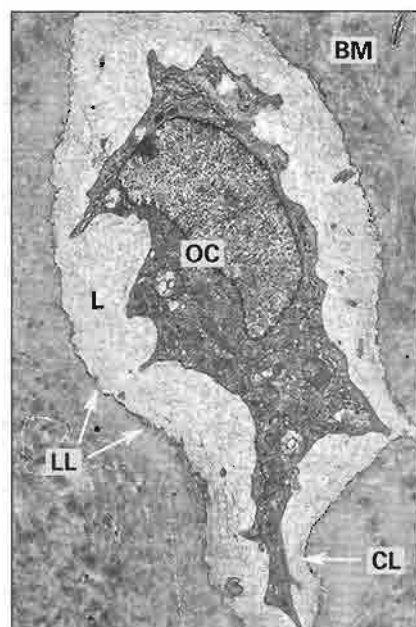


Fig 8-4 Osteocyte (OC) inside a lacunar (L) space. A thin electron-dense layer, the lamina limitans (LL), covers the surface of bone matrix (BM), which was demineralized during tissue preparation. Canaliculi (CL) extending from the lacuna contain bone fluid and osteocyte cytoplasmic processes. (Original magnification $\times 3,600$.)

tion.³⁵ Insulin-like growth factor I (IGF-I), PGE₁, and PGE₂ also stimulate the secretion of bone matrix (Fig 8-3). In the presence of osteoblasts, PGE₂ promotes both osteoclast formation and bone resorption.

Osteoblasts express receptors for vitamin D₃ and PTH, two hormones that activate bone resorption and calcium mobilization. They induce changes in the actin and myosin cytoskeleton and a change in osteoblast shape.³⁶ Contraction of the osteoblast in response to PTH and 1,25-dihydroxy-vitamin D₃ increases the width of the intercellular spaces to expose more of the osteoid surface to the interstitial fluid.^{37,38} The osteoblast retraction induced by PTH appears to be one of the first events in cessation of bone matrix deposition and in triggering a homing response for preosteoclasts. Secretion of collagenase and plasminogen activator is also part of the os-

teoblastic response to PTH, retinoic acid, and vitamin D₃ (see Fig 8-3).^{14,39,40} Interleukin 1 (IL-1), tumor necrosis factor (TNF), and epidermal growth factor (EGF) have been shown to deactivate osteoblasts and to increase the release of colony-stimulating factor 1 (CSF-1) and osteoclast differentiation factor/tumor necrosis factor superfamily 11 (ODF/TNFSF-11), both of which are osteoclast differentiation factors (ODFs), from osteogenic cells (see Fig 8-3).

Osteoblasts promote formation of new blood vessels through secretion of vascular endothelial growth factor (VEGF), a mitogen for endothelial cells.⁴¹ The development of new blood vessels is an essential component of new bone formation and the repair of bone defects.

During bone formation, osteoblasts become entrapped in bone matrix and are transformed into os-

teocytes (Fig 8-4). The precise mechanism or mechanisms that account for the entrapment of osteoblasts are not known. It has been suggested that a slight reduction in bone matrix secretion by an older cell, combined with a steady rate of secretion by neighboring osteoblasts, could lead to encasement of the older cell by matrix.⁴² This theory implies an asynchronous differentiation of osteoblasts along the bone surface.

Others have proposed that the polarity of secretion might shift from an apical to a basolateral mode or that a polymodal form of secretion occurring over the whole cell surface traps the cell in matrix. Another possibility worthy of investigation might be a programmed shift in integrins with binding affinities for bone matrix proteins from a distribution at the apical end of the cell to a distribution over the entire osteoblast surface. In support of this line of thinking it has been shown that osteocytes, but not osteoblasts, stain intensely for CD44, a multifunctional cell surface adhesion molecule with affinities to collagen and fibronectin.⁴³

Osteocytes

Osteocytes are contained in a lacunar space filled with bone fluid, unmineralized collagen fibrils, and proteoglycans. Once they have become fully entrapped in bone matrix, osteocytes exhibit diminished synthetic and secretory capacities. The RER compartment and the Golgi complex are smaller, and secretory granules are rarely present. Lysosomal granules and mitochondria are present in moderate numbers.

The osteocyte develops many cytoplasmic processes, preferentially facing in the direction of the overlying osteoblasts and bone-lining cells, where the nutrient supply is highest.⁴⁴ Individual cell processes occupy small channels, or canaliculi, that are continuous with the lacunar space surrounding the osteocyte (Figs 8-4 and 8-5). The lacunae and canaliculi form a space for the circulation of bone fluid from the deepest osteocytes to the osteoid layer.⁴⁵

A second pathway for a flow of ions and metabolites across bone is an intracellular route through cytoplasmic processes and gap junctions (see Fig 8-5). The gap junctions permit the osteocytes and the cells on the bone surface to interact as a syncytium.

Osteocytes participate in calcium homeostasis. It has been suggested that osteocytes mobilize calcium and other ions from bone matrix and transport them via cell processes and canalicular channels to

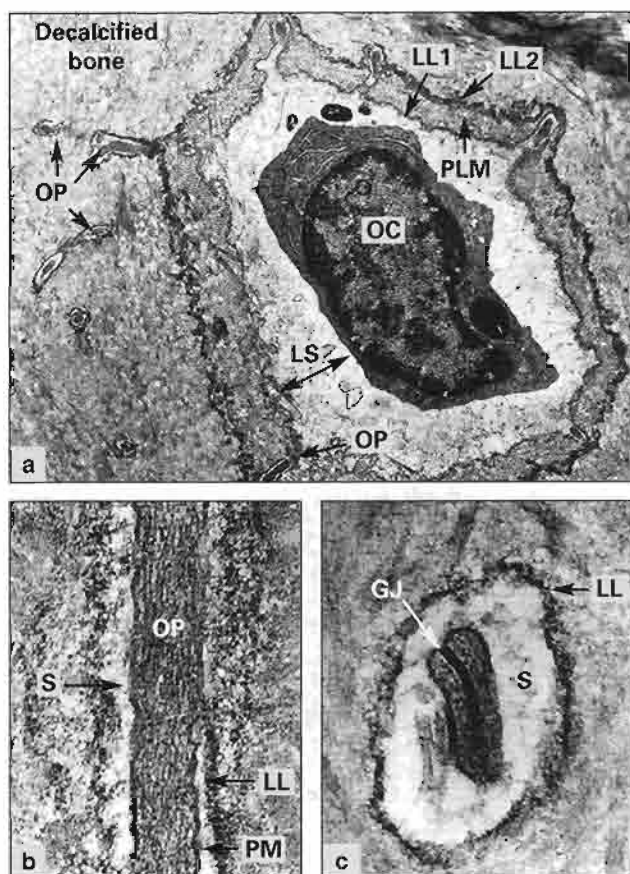


Fig 8-5 (a) Osteocyte (OC) surrounded by lacunar space (LS) and demineralized perilacunar matrix (PLM). Dense lamina limitans lines (LL1 and LL2) border the perilacunar matrix. Osteocyte processes (OP) penetrate the perilacunar matrix into the adjacent bone matrix. (Original magnification $\times 3,600$.) (b) Osteocyte process (OP) in a canalicular space (S) lined by a lamina limitans (LL). The process contains a dense network of cytoplasmic filaments. (PM) Plasma membrane. (Original magnification $\times 39,000$.) (c) Osteocyte processes form gap junctions (GJ). (LL) Lamina limitans; (S) canalicular space. (Original magnification $\times 30,000$.)

osteoblasts (and bone-lining cells) for exchange with general body fluids at the bone surface. The large surface area of mineralized bone that contacts osteocytes and their cell processes, and the potential for cytoplasmic communication via gap junctions, support the concept that osteocytes move calcium ions into and out of bone matrix in response to systemic demands. Large-scale resorption of perilacunar bone matrix (osteocytic osteolysis) is no longer viewed as a normal physiologic response dictated by the requirements of calcium homeostasis.

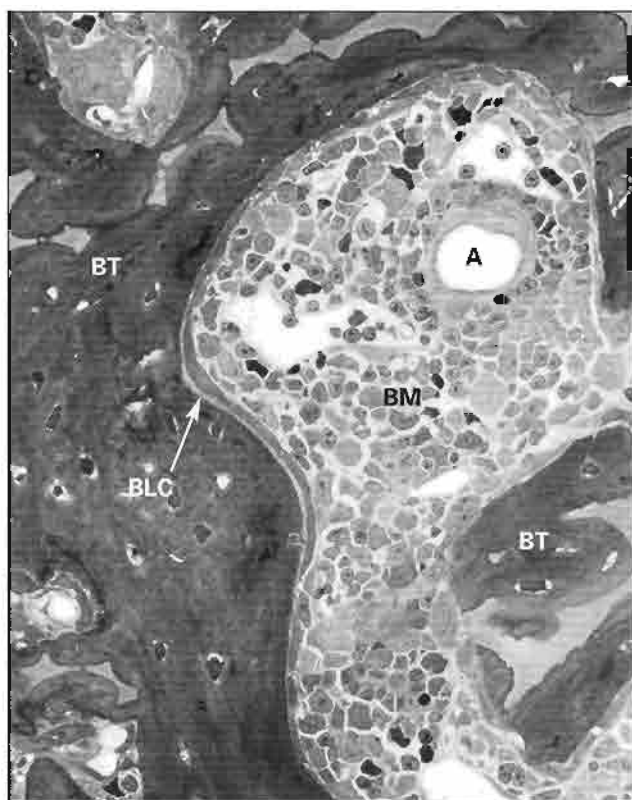


Fig 8-6 Bony trabecula (BT) covered by flat bone-lining cells (BLC) along its endosteal surface facing bone marrow (BM). (A) Arteriole. (Original magnification $\times 280$.)

Advances in bone cell culture technology have made it possible to isolate and culture osteocytes. Future studies of isolated osteocytes will help to identify their role in bone and mineral homeostasis. For example, the finding that isolated osteocytes increase levels of cAMP in response to PTH suggests that osteocytes participate in the overall response of this hormone.⁴⁶

Recent experiments on cultured osteocytes have identified a mechanosensory function for osteocytes in sensing bone fluid flow during bone deformation.⁴⁴ Osteocytes, acting as transducers, convert physical changes into chemical signals that modulate the bone-remodeling activity of local osteoblasts and osteoclasts (see "Response of bone to loading forces," later in this chapter). Acting in this capacity, osteocytes could initiate local alterations of bone shape and mass designed to reduce strains exerted by loading forces.

During bone resorption, osteocytes are liberated from the bone matrix by osteoclasts. This process

occurs directly beneath the osteoclast. The fate of liberated osteocytes is not fully understood. Electron microscopic observations of active bone-resorbing sites indicate, however, that many osteocytes appear to be engulfed by clear zone cytoplasm of osteoclasts.⁴⁷ When completely isolated from the interstitial fluids by the osteoclast, the osteocyte undergoes condensation of nuclear material, cytoplasmic vacuolization, and disintegration. It is not known whether all liberated osteocytes are destroyed in this apoptotic process or whether some survive to return to the osteogenic pool. In fact, it has been suggested that early apoptotic events in aging osteocytes trigger osteoclastic activity and bone turnover. Thus, the eventual fate of all osteocytes is programmed cell death.

In the development of bone trabeculae, as the thickness of bone approaches its physiologic limit, the recruitment of new preosteoblasts to the bone surface is diminished. Under these conditions, whenever a new osteocyte is formed, the remaining osteoblasts must spread over a greater area of the bone surface. Eventually bone formation ceases at that site, and the resting bone surface is covered by extremely flattened bone-lining cells (Fig 8-6).⁴⁸ Between the bone-lining cell and the mineralized bone surface there is no osteoid.

The reduction in osteoblastic production of osteoid is probably regulated by the inability of the deepest osteocytes to obtain adequate nourishment and/or by systemic or paracrine hormonal signals impinging on the osteoblastic layer. The ability of osteocytes to communicate via gap junctions with the osteoblasts, as well as with the bone-lining cells, is probably a key pathway for the transmission of factors regulating and coordinating these changes.

Bone-lining cells

Bone-lining cells extend flat cytoplasmic sheets over the bone surface (see Fig 8-6). It is estimated that 80% of the total bone surface is covered by bone-lining cells. Approximately 20 bone-lining cells line every linear millimeter of resting bone surface. Beneath the bone-lining cell, the osteoid is replaced by a narrow zone of unmineralized connective tissue matrix. Bone-lining cells act as gatekeepers, protecting the bone surface from osteoclasts, regulating the ionic composition of bone fluid, and regulating the initiation of new bone formation or bone resorption.^{48,49}

Bone-lining cells contain a relatively small number of organelles. Mitochondria, free ribosomes, RER, and Golgi cisternae are located adjacent to the flat-

tened nucleus of the bone-lining cell. Bone-lining cells are not connected by zonula occludens junctions; thus there is no tight cytoplasmic barrier between bone and the general body fluids. Despite the lack of occluding junctions, differences in ionic composition exist between bone fluid and the interstitial fluids.^{50,51} Differential ion concentrations between these two compartments are thought to be maintained by a combination of cell membrane transport and charge restriction mediated by fixed proteoglycans in the lamina limitans.

Bone-lining cells can be stimulated to incorporate thymidine, divide, and give rise to osteoblasts. The osteoprogenitor capacity of bone-lining cells is important in responding to increased strain and in forming a fracture callus during bone repair.⁵²

Lamina limitans and cement lines

All inactive bone surfaces are covered by a thin, densely stained lamina limitans (see Figs 8-5a to 8-5c). In electron micrographs of demineralized bone, the lamina limitans consists of dense granular matrix similar to that of a cement line. Osteopontin is a major component of cement lines and the lamina limitans.^{53,54} Because of its ability to bind to mineral as well as to cellular integrins via its tripeptide sequence of arginine-glycine-aspartic acid (RGD), osteopontin appears well suited to act as an all-purpose adhesive between cells and bone at the lamina limitans and between old and new bone segments at cement lines. A lamina limitans is not present over active bone surfaces, such as beneath osteoblasts and at osteoclast ruffled borders.

Basophilic cement lines demarcate successive layers of new bone formation. These lines represent thin layers of organic matrix, rich in glycoproteins and proteoglycans that bind the collagen fibrils of the new matrix to that of the old bone matrix. Ultrastructurally, the matrix of the cement line is characterized by globular accretions of dense material. It has been suggested that these globular accretions are secreted by osteoblasts just prior to formation of new bone and are not formed by nonspecific precipitation of plasma and tissue proteins over resting bone surfaces.^{53,55}

Components of the Bone Matrix

The organic composition of bone is made up of collagenous and noncollagenous proteins and proteoglycans. Growth factors (CSF-1) and chemokines

(CK β -8), regulators of osteoclast formation, are also deposited in the bone matrix.^{56,57} Type I collagen is the major component of the organic matrix of bone. It constitutes about 90% of the bone protein and it provides the structural framework to support the mineral phase. The structure of type I collagen is discussed in chapter 6, and its role in biologic mineralization is discussed in "Biologic mineralization of tissues," later in this chapter.

The noncollagenous proteins function in bone matrix mineralization, cellular adhesion, and regulation of bone cell activity during coupling of bone formation and resorption.⁵⁸ They have been localized and quantified in human bone tissue by immunohistochemistry.⁵⁹

Osteocalcin

Osteocalcin is a low-molecular weight protein containing three α -carboxyglutamic acid residues per molecule (also called *GLA protein*). Osteocalcin is one of the most abundant noncollagenous proteins of bone matrix. Vitamin K is required for the synthesis of the α -carboxyglutamic acid residues. These residues provide calcium-binding sites that are believed to play a role in bone matrix mineralization or in the regulation of crystal growth (see "Biologic mineralization of tissues").

The role of osteocalcin in bone mineralization is supported by the observation that osteocalcin messenger ribonucleic acid (mRNA) is localized in osteoblasts and simultaneously in the mineralized bone matrix. Osteocalcin has since been localized over the mineralized portion of bone and in acellular cementum.^{53,54} Serum levels of osteocalcin have been measured as an index of osteoblastic activity. Osteocalcin and the chemokine CK β -8 act as chemoattractants for preosteoclasts and may be essential for osteoclast differentiation.^{57,60} In vitro studies of the response of osteoclast-like giant cells to osteocalcin indicate that it promotes the adhesion and spreading of these cells through increased secretion of osteopontin, fibronectin, and bone sialoprotein, and the formation of focal adhesions.⁶⁰

Bone sialoprotein

Bone sialoprotein, which has a molecular mass of about 33,000 kDa, contains the RGD tripeptide sequence, a motif contained in attachment proteins that interact with cell surface integrins. Bone sialoprotein contains a stretch of 10 glutamic acid residues, providing a negatively charged domain with

high calcium-binding potential. Thus bone sialoprotein can bind tightly to hydroxyapatite as well as to cells. Immunocytochemical localization of bone sialoprotein showed that it is not found in osteoid but is restricted to the mineralized bone matrix.¹⁵

Calcium-binding proteins, such as bone sialoprotein and osteopontin, have been shown to inhibit mineral deposition when present in solution. However, when bound to a solid substrate they can act as promoters of mineral deposition.⁶⁸ It has been proposed that the association of osteocalcin and/or bone sialoprotein with collagen fibrils creates locally high concentrations of calcium, leading to precipitation of mineral (see "Biologic mineralization of tissues"). Binding studies have shown that bone sialoprotein has an affinity for the hole zone of collagen fibrils.⁶¹

Immunocytochemical localization of bone sialoprotein has revealed high concentrations of the protein at the epiphyseal-metaphyseal border during endochondral bone formation.⁶² This localization is consistent with a role in binding osteoclasts and osteoblasts to the mineralized cartilage matrix. Bone sialoprotein increases osteoclastic resorption by promoting greater adhesion of osteoclasts to bone matrix molecules.⁶³

Osteopontin

Osteopontin is another charged protein, similar to bone sialoprotein, that is expressed in differentiating bone cells.^{53,64,65} The regulation and function of osteopontin in osteoblasts are subjects of a recent review by Sodek et al.⁶⁶ Osteopontin contains several serine phosphorylation sites and a stretch of nine negatively charged aspartic acid residues that bind calcium. Osteopontin also has an RGD sequence with specificity toward cell surface integrins (in this case to the vitronectin receptor, $\alpha v \beta 3$).

Osteopontin is concentrated in small globular deposits in bone matrix and in the lamina limitans at the bone surface, suggesting that it plays a role in bone mineralization and in the attachment of osteoblasts and osteoclasts to bone matrix.^{53,64,67} Osteopontin has been shown to form cross-links to fibronectin through the catalytic action of bone matrix transglutaminase.⁶⁸ The concentration of osteopontin in the cement lines that lie between old and new bone segments indicates that it acts as a biologic matrix-bonding agent. Osteopontin is expressed by a variety of cell types and is found in many soft tissues, suggesting that it may have a role in soft tissue organization. Its significance in development may be re-

lated to its increased expression during mesenchymal cell migration.⁶⁵ Recent studies have shown that osteopontin, also called *early T-lymphocyte activation 1* (Eta-1), plays a key role in activating cell-mediated immunity.⁶⁹

Osteonectin

Osteonectin (also known as *secreted protein, acidic and rich in cysteine* [SPARC]) is the most abundant noncollagenous protein in bone. It is expressed by osteoprogenitor cells, osteoblasts, and newly formed osteocytes. Osteonectin is a 32-kDa protein with calcium- and collagen-binding domains.⁷⁰ Although osteonectin has been proposed to have a role in the initiation of mineralization of bone matrix, its exact function is still unclear. Numerous cells of soft tissues, such as periodontal ligament (PDL) fibroblasts and endothelial cells, also produce osteonectin. As a result of its ability to bind various collagens and substrate adhesion molecules, osteonectin may have a generalized function in a calcium-mediated organization of extracellular matrices.⁵⁸

Biglycan and decorin

Two proteoglycans found in most connective tissues, biglycan and decorin, are also contained in bone matrix.⁷¹

Growth factors

Growth factors such as bone morphogenetic proteins, transforming growth factor β , colony stimulating factor 1, granulocyte colony-stimulating factor, basic fibroblast growth factor (bFGF), and insulin-like growth factor are secreted by osteoblasts.^{56,72-76} These factors may act immediately in an autocrine or paracrine manner or may be incorporated in bone matrix for later action. During osteoclastic bone resorption, these growth factors are released and activated to exert autocrine and paracrine regulatory effects on osteogenic cells and osteoclasts. The specific actions of various growth factors and cytokines on bone cells are described in later sections.

Development and Function of Osteoclasts

Osteoclasts are highly specialized for resorption of bone mineral and matrix through the coordinated secretion of hydrogen ions and proteolytic enzymes.⁷⁷

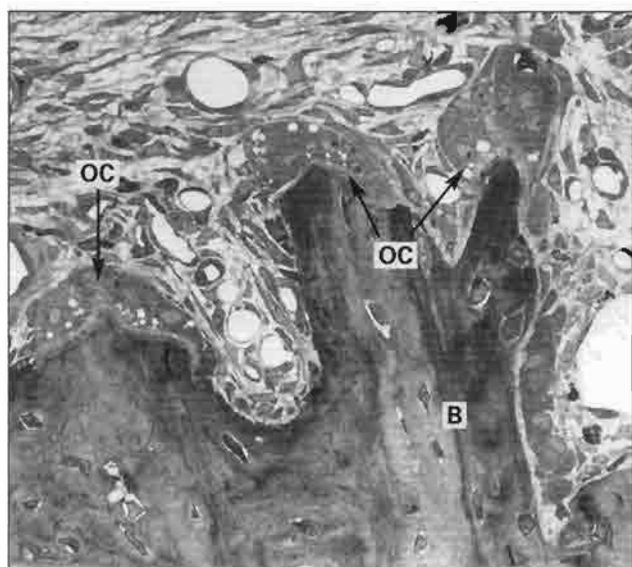


Fig 8-7 Osteoclasts (OC) resorbing the ends of alveolar bone spicules (B) adjacent to a developing tooth. (Original magnification $\times 280$.)

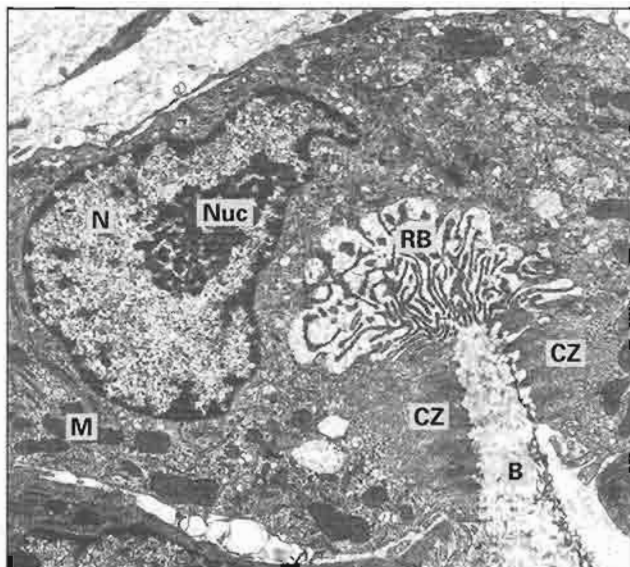


Fig 8-8 Small osteoclast situated over a narrow piece of bone (B). Note the ruffled border (RB) and the adjacent clear zone (CZ) cytoplasm, which is closely adapted to the bone. (M) Mitochondria; (N) nucleus; (Nuc) nucleolus. (Original magnification $\times 4,600$.)

Although most osteoclasts are large multinucleated cells, there are reports of mononuclear osteoclasts.⁷⁸ In tissue sections, osteoclasts are identified by their multinucleated appearance, expression of calcitonin receptors, and positive staining for tartrate-resistant acid phosphatase (TRAP) (Figs 8-7 to 8-9).^{79,80}

Osteoclastic bone resorption is called on for the alteration of bone shape and mass in adaptation to physical stresses exerted on the skeleton. Under physiologic conditions, osteocytes and bone-lining cells, not osteoclasts, fine-tune the interstitial fluid and plasma calcium levels.

In pathologic inflammatory conditions, such as periodontal disease, osteoclastic activity is initiated in response to stimulatory factors produced by cells of the inflammatory infiltrate. Prostaglandin E_2 and osteoclast-activating factor (now regarded to be interleukin 1β) are two substances generated in inflamed tissues that exhibit potent osteoclastic stimulatory activity.

In bone turnover, the resorption phase is followed by (coupled to) a subsequent formation phase. The resorption phase of the bone formation-resorption cycle lasts about 8 to 10 days. Presumably this is the life span of the multinucleated osteoclast. Morphologic and biochemical evidence indicates that old os-

teoclasts undergo apoptosis, a process involving condensation of chromatin and DNA fragmentation.^{81,82} Interleukin 1β and CSF-1 prolong the survival of osteoclasts in vitro by suppressing apoptosis-promoting interleukin 1β -converting enzyme proteases.⁸²

Microcinematography of live osteoclasts reveals large, motile cells capable of migrating over the bone surface.⁸³ Bone resorption takes place beneath stationary osteoclasts in regions of the cell characterized by intense cytoplasmic motion and vesicular traffic. Following the removal of bone and the creation of a resorption pit, the osteoclast may move laterally to begin a new resorption pit.

In its multinucleated and fully differentiated form, the osteoclast is easily identified in histologic sections. Situated on the bone surface, it occupies a concavity (Howship's lacuna) created by its polarized secretory activity, or it may cap the resorbing surface of small bone spicules (see Figs 8-9a to 8-9c).

An enlarged surface area created by plasma membrane infoldings, the ruffled border, characterizes the secretory or apical surface directed toward the bone (see Figs 8-8 and 8-9).⁸⁴ In routine histologic sections, the ruffled border appears striated and lightly stained. The presence of a ruffled border is an indication that the osteoclast is actively engaged in bone resorption.⁸⁴ Large osteoclasts may

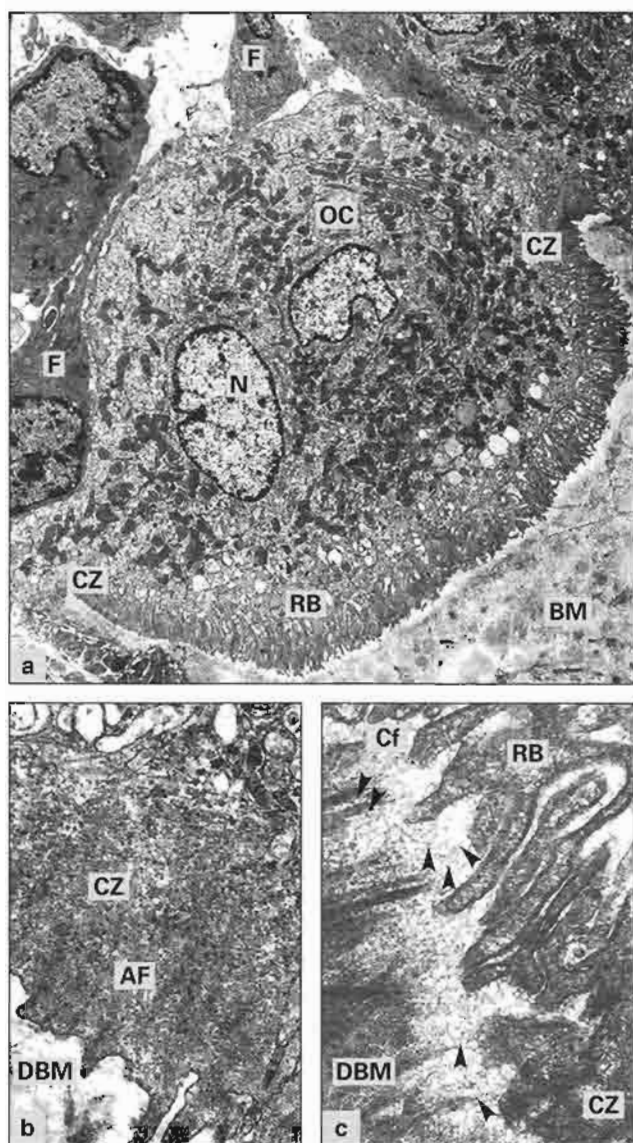


Fig 8-9 (a) Osteoclast (OC) within shallow resorption pit (Howship's lacuna). The apical surface is characterized by a large ruffled border (RB) flanked by clear zones (CZ). (BM) Bone marrow; (F) fibroblast; (N) nucleus. (Original magnification $\times 3,500$.) (b) The clear zone (CZ) contains a dense network of thin actin filaments (AF) and various actin-binding proteins. (DBM) Demineralized bone matrix. (Original magnification $\times 18,000$.) (c) The ruffled border (RB) is the site of membrane infoldings to increase the surface area in contact with demineralized collagen fibrils (Cf) that are undergoing destruction (arrowheads) beneath the RB. (CZ) Clear zone; (DBM) demineralized bone matrix. (Original magnification $\times 22,000$.)

have several ruffled border specializations or resorptive sites along their apical surface.

At the electron microscopic level, the cell membrane of the ruffled border is observed to contain numerous closely spaced protein particles that project into the cytoplasm. These membrane-associated particles have been shown to be transmembrane proton pump complexes responsible for generating the acidified milieu of the bone resorption compartment located beneath the ruffled border.⁸⁵

Each ruffled border is surrounded by a clear zone (or sealing zone), a cytoplasmic area rich in cytoplasmic actin filaments and devoid of major cytoplasmic organelles (see Fig 8-9a to 8-9c).⁸⁴⁻⁸⁷ Through close adaptation of the cell surface to the bone matrix, the clear zone establishes a seal between the bone resorption compartment and the interstitial fluid. Although the structure of the clear zone was first studied at the ultrastructural level, the recent use of fluorescent antibodies directed against actin have elegantly demonstrated its overall shape as well as its specialization for attachment to bone.^{87,88}

The clear zone demarcates the apical plasma membrane (ruffled border) from the basolateral plasma membrane.^{85,89} The basolateral membrane is specialized for interaction with the adjacent connective tissue microenvironment.⁸⁹ It contains receptors for hormones, cytokines, and other factors that have a controlling influence over osteoclastic activity. It is the site of several membrane transport systems needed for maintaining a physiologic electrolyte balance inside the cell.

Although mononuclear osteoclasts have been described, the typical osteoclast is multinucleated.^{78,90} Most commonly, the cell contains two to 20 nuclei positioned within the basal cytoplasm (away from the bone). Prominent nucleoli are present. The RER is distributed throughout the basolateral cytoplasm in the form of short, narrow, and branching cisternal profiles. Golgi complexes are relatively abundant and well developed.⁸⁵ Osteoclast Golgi complexes are characterized by long, flattened stacks of cisternae positioned close to and parallel to the each of the nuclei. It is assumed that each nucleus is associated with a cytoplasmic domain containing a Golgi complex, a centriole pair, and microtubule organizing centers.

Metalloproteinases and lysosomal enzymes are packaged in relatively small secretory granules in the Golgi complex. The structure of the secretory granules is best observed in osteoclasts that have been exposed to microtubule inhibitors. Under these conditions, the granules are not transported to the apical

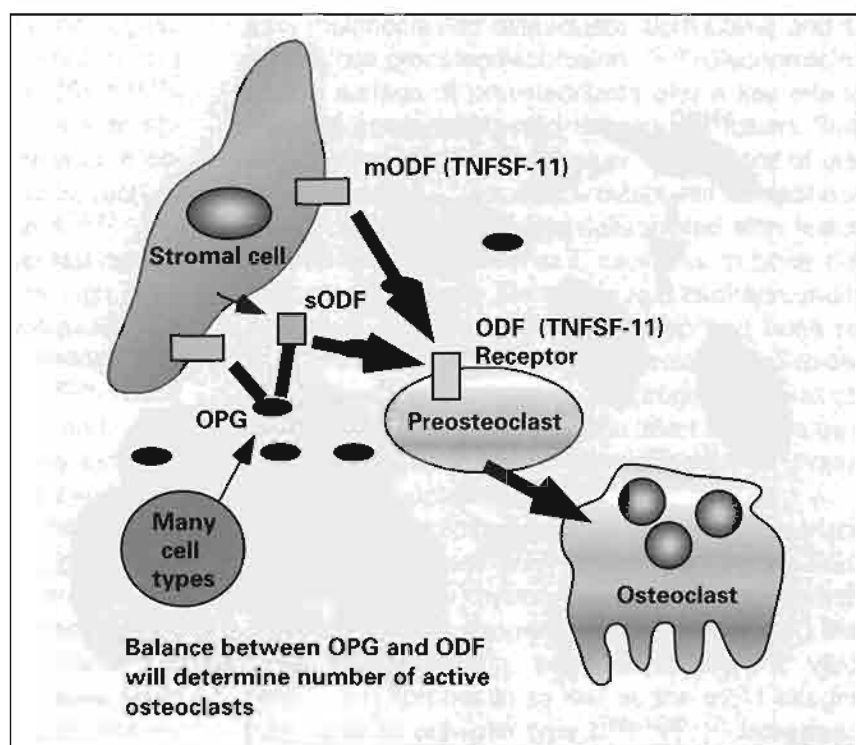


Fig 8-10 Positive regulation of osteoclast formation through binding of osteoclast differentiation factor/tumor necrosis factor superfamily 11 (ODF/TNFSF-11) to its receptor and negative regulation of osteoclast formation by the interaction of osteoprotegerin (OPG) with ODF. (mODF) Membrane-bound osteoclast differentiation factor; (sODF) soluble osteoclast differentiation factor.

membrane and they accumulate in the cytoplasm in large numbers. The granules have short, cylindrical bodies and contain material of moderate electron density. They resemble newly formed lysosomal granules observed inside monocytes and macrophages.

Osteoclasts contain the highest concentration of mitochondria of any cell type. Mitochondria generate ATP, required by the ruffled border H^+ (ATPase) pump and many other membrane active transport systems, as well as for generating carbon dioxide, which is used in the carbonic anhydrase-catalyzed production of hydrogen ions. Other cytoplasmic organelles include secondary lysosomes, vacuoles, and endosomes. The vacuoles are numerous adjacent to the ruffled border, accounting for the "foamy" nature of this region of the cell at the light microscopic level.

Microcinematography of resorbing bone surfaces has shown that large osteoclasts may have more than one ruffled border zone and that osteoclasts are highly mobile cells. A single osteoclast may create multiple resorption pits by migrating from site to site or by activating several ruffled border zones. It has been proposed that the migration of osteoclasts might result from chemotaxis in response to the secretion of CSF-1 by adjacent osteoblasts.⁹¹

Origin and development

Osteoclasts arise from hematopoietic stem cells that give rise to the monocyte and macrophage cell line (Figs 8-10 and 8-11).^{90,92-95} Under appropriate conditions, bone marrow-derived monocyte and macrophage colony-forming cells, peripheral blood monocytes, and tissue macrophages may undergo osteoclastic differentiation.⁹⁶⁻⁹⁸ Factors that regulate the development of functional osteoclasts include osteoblastic stromal cell factors, such as monocyte colony-stimulating factor 1, osteoclast differentiation factor, interleukins, vitamin D_3 , tumor necrosis factor α , and contact with mineralized bone particles containing osteocalcin.⁹⁸⁻¹⁰⁶

Osteoclast differentiation factor is expressed as a membrane-bound protein (mODF) or in soluble form (sODF) by osteoblasts or stromal cells (see Fig 8-10).¹⁰⁷ Osteoclast differentiation factor is a member of the superfamily of tumor necrosis factors and in the most recent nomenclature has been named *TNFSF-11*.¹⁰⁸ The terms *TNF-related activation-induced cytokine receptor* (TRANCE) and *receptor activator of nuclear factor κB ligand* (RANKL), found in the older nomenclature, are synonymous with ODF/TNFSF-11.

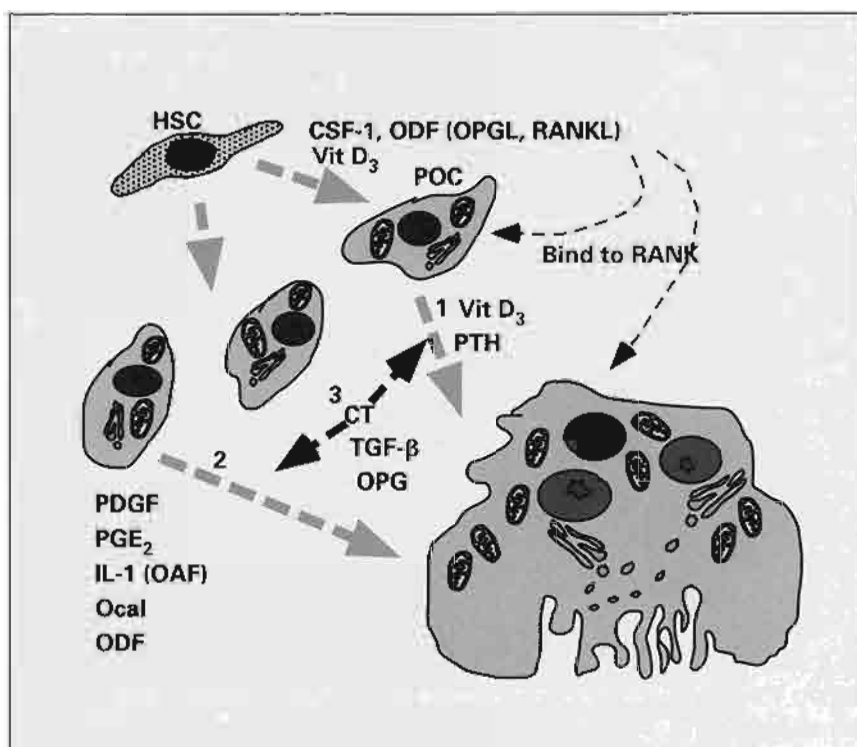


Fig 8-11 Development of a preosteoclast (POC) from hematopoietic stem cells (HSC) under the influence of monocyte colony-stimulating factor 1 (CSF-1) and 1,25-dihydroxy-vitamin D₃ (Vit D₃). Systemically acting substances, such as platelet-derived growth factor (PDGF), prostaglandin E₂ (PGE₂), and osteoclast-activating factor (OAF), stimulate the formation of multinucleated osteoclasts from circulating mononuclear preosteoclasts. Osteocalcin (Ocal), present in mineralized bone, is necessary for osteoclast formation. Calcitonin (CT) and transforming growth factor β (TGF- β) act to block the formation of osteoclasts. (IL-1) Interleukin 1; (ODF) osteoclast differentiation factor; (OPGL) osteoprotegerin ligand; (PTH) parathyroid hormone; (RANK) receptor activator of nuclear factor κ B; (RANKL) receptor activator of nuclear factor κ B ligand.

When ODF/TNFSF-11 binds to its receptors on preosteoclasts, it promotes osteoclast formation (see Fig 8-10).^{109,110} Osteoprotegerin (OPG) is a circulating protein that inhibits osteoclast formation by binding mODF/TNFSF-11 expressed on osteoblasts and stromal cells, thereby preventing the stimulatory cell-to-cell interaction with preosteoclasts.^{95,109,111} Recent *in vitro* studies have demonstrated that sODF/TNFSF-11 (also known as *osteoprotegerin ligand*), in combination with CSF-1, stimulates osteoclast development from peripheral blood cell precursors by binding to its receptor (see Figs 8-10 and 8-11).^{110,112,113} Activation of the ODF receptor increases the expression of TRAP, β 3 integrins, cathepsin K, and calcitonin receptors on preosteoclasts.^{95,110,112} Mice that lack CSF-1 or ODF/TNFSF-11 develop osteopetrosis and exhibit delayed tooth eruption and defects in T- and B-cell differentiation. In summary, OPG (negative regulator) and ODF/TNFSF-11 (positive regulator) control osteoclastogenesis by interacting with appropriate receptors on cells of the monocyte and macrophage cell line.

The C-terminal fraction of PTH has also been shown to increase osteoclast formation and the bone-resorbing activity of mature osteoclasts. Rodan¹¹⁴ made the interesting point that osteoclastic bone resorption and inflammation are closely re-

lated, in that both are activated by injury and are regulated by similar factors.

In the absence of osteoclastic activity, bones become abnormally dense—a pathologic condition known as *osteopetrosis* or *marble-bone disease*. The study of osteopetrotic rats and mice has provided important clues to the origin and development of osteoclasts. Early experiments suggested that the defect in osteopetrotic mice was a lack of mononuclear osteoclast precursors. This view was supported by the observation that the osteopetrotic condition could be partially cured by the infusion of bone marrow and spleen cells from normal littermates. These results were also taken to be proof of the bloodborne nature of the osteoclast precursor.

More recent studies have shown that the osteopetrotic (*op/op*) mouse has a mutation in the gene encoding colony-stimulating factor 1.⁹⁹ Injection of normal CSF-1 (monocyte CSF) into osteopetrotic mice leads to the formation of osteoclasts and a partial cure.^{115,116} It has also been established that the osteoblasts are responsible for producing CSF-1 and that contact between osteoblasts and osteoclast precursors, mediated by CSF-1 and its receptor, promotes osteoclast development. It now appears that the *op/op* osteopetrotic mouse condition is not the result of an intrinsic lack of osteoclast precursors but

rather in an abnormality of the osteoblastic regulation of osteoclast differentiation, manifested in part by the production of biologically inactive CSF-1.^{99,115}

Other forms of osteopetrosis result from an absence of TNFSF-11 or from inherent defects in osteoclast precursors and mature osteoclasts, such as mutations of *c-src* protein and cathepsin K.^{95,117}

At the microscopic level, the preosteoclast resembles a mononuclear cell characterized by high numbers of mitochondria, a moderately well-developed Golgi apparatus, low numbers of RER cisternae, and only small stores of glycogen particles.^{118,119} These cells differ in appearance from monocytes and newly differentiated macrophages because they lack the numerous fingerlike cytoplasmic processes and coated pits and vesicles found in monocytes. Monocytes and macrophages stain strongly for nonspecific esterase but very poorly for TRAP and osteopontin, while the opposite is true for preosteoclasts.¹¹⁸ Osteopontin- and TRAP-positive preosteoclasts are usually present in the vicinity of sites of active bone resorption. Preosteoclasts are also found in perivascular locations adjacent to endosteal and periosteal bone surfaces. Preosteoclasts synthesize osteopontin, $\alpha\text{v}\beta 3$ integrins, cell surface receptors for CSF-1, ODF/TNFSF-11, and calcitonin.^{118,120,121}

Although the conditions that target a bone surface for resorption have not been fully identified, one condition that must be met is the exposure of the mineralized bone to the interstitial fluid. This is accomplished by contraction of osteoblast and/or bone-lining cells, which increases the width of intercellular spaces. This is followed by the degradation of the osteoid and/or connective tissue layer overlying the mineralized bone surface by osteoblasts or activated macrophages (see Fig 8-3).¹²²

Factors responsible for increasing the number and activity of osteoclasts, such as PTH, 1,25-dihydroxyvitamin D₃, and IL-1, do so by acting directly on osteogenic cells, causing cytoplasmic contraction and the secretion of collagenase, tissue plasminogen activator, and osteoclast differentiation factor (see Fig 8-3).¹²³ The expression of urokinase plasminogen activator by osteoblasts leads to focal generation of plasmin, which in turn activates matrix metalloproteinases and the degradation of the nonmineralized osteoid matrix.¹²⁴ Degradation of osteoid and collagen matrix releases osteocalcin and other factors, such as the chemokine CK β -8, that are chemotactic for preosteoclasts.⁵⁷

Contact with the mineral phase, particularly contact with osteocalcin, triggers terminal osteoclastic differentiation and mononuclear osteoclast fusion to

form multinucleated osteoclasts. Both CSF-1 and IL-1 stimulate preosteoclast fusion.^{125,126} Glycoproteins on the surface of preosteoclasts play a key role in cell-to-cell recognition and subsequent fusion. Substances that bind to mannose on the surface of preosteoclasts are able to block osteoclast formation in vitro.¹²⁷ Cell-to-cell adhesion associated with fusion of preosteoclasts requires E cadherin. In bone cell cultures treated with antibodies to E cadherin, multinucleated osteoclasts fail to develop and bone resorption is inhibited. Cadherin-mediated cell-to-cell interactions between osteoclast progenitors and osteoblasts/stromal cells have also been shown to be a prerequisite to osteoclast formation in a mouse model of osteoclastogenesis.¹²⁸

Contact of preosteoclasts with the bone surface permits plasma membrane integrins to bind to RGD peptides of bone matrix proteins.^{129,130} Osteoclasts express the $\alpha\text{v}\beta 3$ integrin (vitronectin receptor) that binds to osteopontin, bone sialoprotein II, vitronectin, and fibronectin as well as the $\alpha 2\beta 1$ integrin that binds to collagen type I.^{120,129,131-133} Integrin-to-matrix binding serves the dual function of cell-to-cell substrate attachment and cell activation via calcium-mediated cytoplasmic signaling.

Cytoskeleton components that participate in stabilizing the initial osteoclast-to-matrix attachment include vinculin, talin, integrins, and actin. These cytoplasmic molecules associate to produce focal anchorage of the cytoskeleton in the same manner as in the formation of focal adhesions in other connective tissue cells. Punctate focal adhesions in osteoclasts take the form of small, circular specializations called *podosomes* (Fig 8-12).^{87,134} In migrating osteoclasts, the podosomes are found predominantly in the leading edge, while in resting osteoclasts they appear evenly distributed around the periphery of the cell.⁸⁸

Reorganization of the components of the podosomes forms a circumferential actin-, integrin-, and cadherin-rich clear zone isolating the ruffled border and the bone resorption compartment at the initiation of a bone-resorbing cycle (see Fig 8-12).^{87,88,134,135} The seal between the clear zone and the bone occurs between mineral and cadherins in the plasma membrane.¹³⁶ Treatment of osteoclasts with peptides that interfere with cadherins leads to loss of clear zone actin rings and deactivation of osteoclasts.¹³⁶

Many substances can block cytoskeletal assembly, disrupt podosomal structure and clear zone formation, and thereby interfere with osteoclastic bone resorption.¹³⁷ Actin-binding proteins with Src homol-

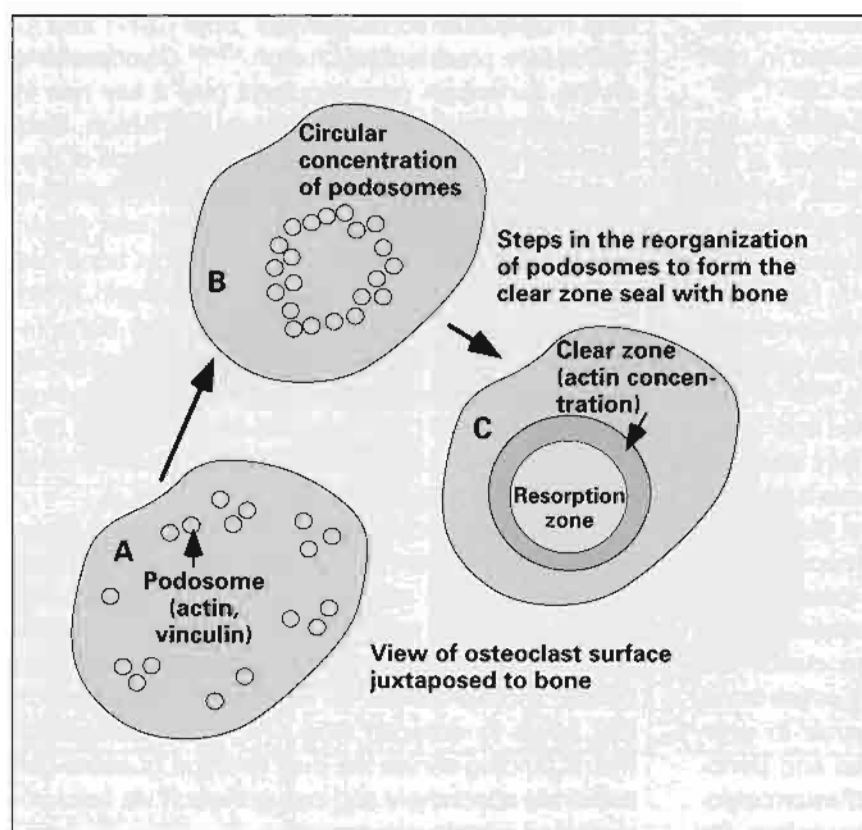


Fig 8-12 Phases in the reorganization of (A) punctate matrix attachment specializations (podosomes) rich in vinculin and actin. (B) Circular concentration of individual podosomes occurs just prior to the resorption phase, characterized by the rearrangement of actin cytoskeleton to form the clear zone. (C) At the clear zone, the plasma membrane is forced into close contact (less than 15 nm) with the bone mineral phase to form a seal between the resorption zone and the interstitial space. (Adapted from Väänänen and Horton with permission from The Company of Biologists.⁸⁷)

ogy domains and focal adhesion kinases are localized to osteoclast focal adhesions.^{138,139} Thus, the attachment of osteoclasts to the bone substratum may regulate the resorptive function.^{138,140} This hypothesis is supported by the observation that the inhibition of focal adhesion kinase by tyrosine kinase inhibitors disrupts osteoclast podosomes and interrupts bone resorption.¹⁴⁰ Additionally, it has been shown that the formation of zones of attachment facilitates (and perhaps dictates) the establishment of apical and basolateral domains in the plasma membrane. The importance of specific cell-to-matrix attachment in osteoclast function is supported by the fact that small peptides with RGD sequences bind to integrins and prevent the formation of osteoclasts.¹⁴¹⁻¹⁴³ Furthermore, antibodies to $\alpha v \beta 3$ block osteoclast formation and bone resorption.¹⁴⁴

Secretory function

Cytoplasmic polarity, established during the formation of the clear zone at the bone surface, directs microtubule-mediated transport of secretory granules toward the ruffled border, creating a resorption com-

partment (Figs 8-9 and 8-13).^{85,89} Granule fusion with the cell membrane and subsequent enzyme release occurs at the ruffled border. The precise mechanisms responsible for establishing and maintaining the apical secretory domain of the osteoclast surface have yet to be defined. Secretion granules in the Golgi complex contain cathepsin enzymes and metalloproteinases. Cytochemical studies have shown that TRAP is present in lysosomes and vacuoles adjacent to the ruffled border.⁷⁹ In contrast, tartrate-sensitive acid phosphatase activity is localized in Golgi cisternae and lysosomes. Although TRAP is not entirely specific for osteoclasts, it is a useful marker for osteoclast identification in histologic sections. Cells of the osteogenic cell line do not express TRAP.

Immunocytochemical procedures have localized cathepsins B and D in cytoplasmic vacuoles near the ruffled border and cathepsins E, S, and L in the extracellular space adjacent to the ruffled border and in contact with collagen fibrils.^{145,146} Human osteoclast DNA contains genes encoding cathepsin K, a novel cysteine protease, homologous to cathepsins S and L.¹⁴⁷ Substrates susceptible to proteolysis by cathepsin K include bone collagen and osteo-

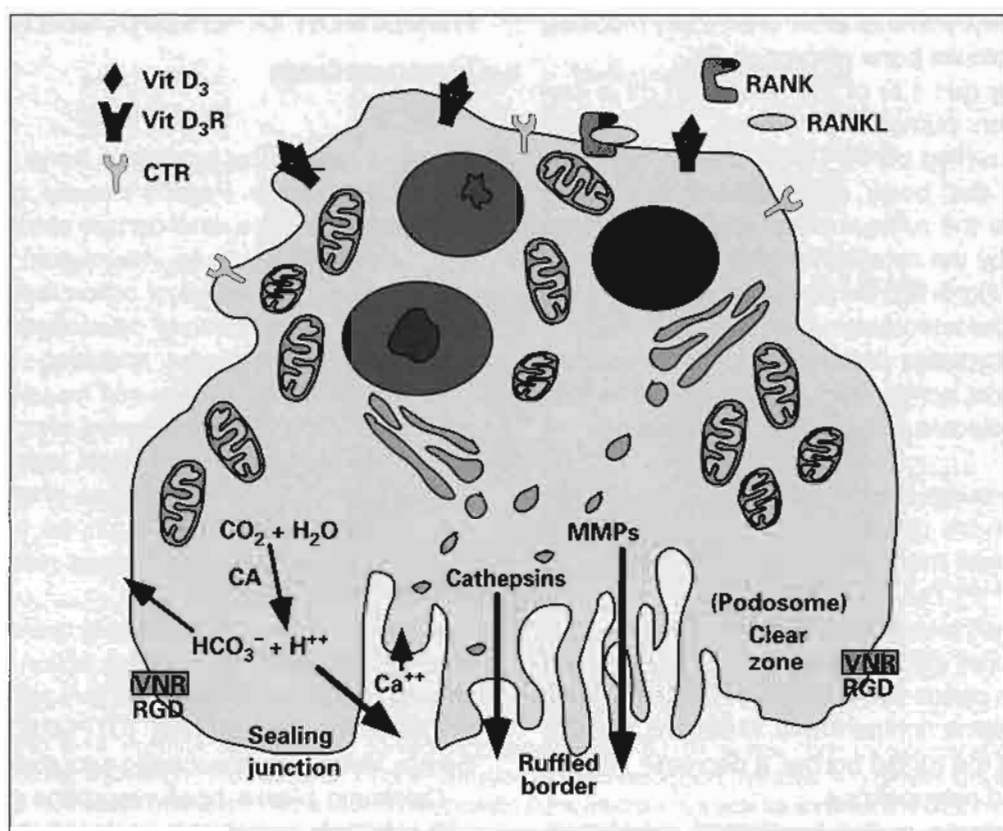


Fig 8-13 Apical surface of the active osteoclast, the site of the secretion of metalloproteinases (MMPs) and cathepsins that degrade the organic matrix of bone. Protons secreted into the extracellular milieu lower the pH, leading to the dissolution of bone mineral. Carbonic anhydrase (CA) converts carbon dioxide to carbonate-releasing hydrogen ions to supply proton pumps located in the membranes of the ruffled border. Vitronectin receptors (VNR), associated with cytoplasmic actin, talin, and vinculin, form attachments to matrix proteins that contain arginine-glycine-aspartic acid (RGD) sequences. At the clear zone, a seal is formed between the plasma membrane and the bone mineral. Calcium enters the cytoplasm via a calcium sensor at the apical surface. High levels of cytoplasmic calcium act to deactivate the resorptive activity of the osteoclast. The basolateral surfaces contain calcitonin receptors (CTR) and vitamin D₃ receptors (Vit D₃R). Binding of vitamin D₃ (Vit D₃) to its receptors in the absence of calcitonin stimulates resorptive activity. (RANK) Receptor activator of nuclear factor κ B; (RANKL) receptor activator of nuclear factor κ B ligand. (Adapted from Blair with permission from John Wiley & Sons.¹⁴⁸)

nectin.^{148,149} Mutations in the gene encoding cathepsin K lead to decreased bone resorption and pyknodysostosis, a form of osteochondrodysplasia, leading to short stature.¹¹⁷ The dissolution of the mineral phase in the acidic microenvironment below the ruffled border exposes collagen fibrils to the enzymatic attack of cathepsins B, K, and L, known to degrade native collagen at acid pH.^{145-147,149-151} Cathepsin K, apparently a specific product of osteoclasts, is concentrated at the ruffled border.¹⁴⁷ Cathepsin E is a nonlysosomal proteinase that localizes in osteoclast endocytic vacuoles and ruffled borders. It is capable of degrading collagen at low pH.¹⁵²

Osteoclasts also express matrix metalloproteinases. Gelatinase A (MMP-2), gelatinase B (MMP-9), stromelysin (MMP-3), and collagenase (MMP-1) have been localized in osteoclasts and are known to participate in bone resorption.^{151,153,154} Matrix metalloproteinase 9 is able to digest collagen types IV and V as well as to depolymerize collagen fibrils by attacking the NH₂ terminals of type I collagen molecules.¹⁵⁵ Although the precise sequence of enzyme activations and matrix degradative steps effected by osteoclastic cathepsins and MMPs is still unresolved, it has been proposed that cysteine proteinases act first, at low pH, followed by MMPs.¹⁵³ Inhibition of

MMPs with doxycycline or other chemically modified tetracyclines blocks bone resorption.¹⁵⁶

The acidity (pH 4.5) of the resorption pit is created by proton pumps (H^+ -ATPase) in the membranes of the ruffled border (see Fig 8-13).^{86,157,158} Attachment to the bone surface stimulates acid extrusion from the ruffled-border region.¹⁵⁹ Protons are supplied by the catalytic activity of carbonic anhydrase (see Fig 8-13). Carbonic anhydrase II is expressed in preosteoclasts and osteoclasts.¹⁶⁰⁻¹⁶³ This enzyme hydrates carbon dioxide, a by-product of mitochondrial activity, to carbonic acid. The carbonic acid molecule ionizes into carbonate and hydrogen ion.

The proton pump is electrically coupled to a chloride channel in the ruffled border that offsets the increased electrical membrane potential generated by the H^+ pump.^{157,158} The chloride channel of the ruffled border has been identified as *ClC-7*, a member of the chloride channel gene family.¹⁵⁷ It is transported, along with the proton pump, to the ruffled border via the late endosome compartment. Mutations in *ClC-7* lead to loss of the ruffled border, a decrease in bone resorption, and osteopetrosis.

Two exchangers in the basolateral membrane, Na^+H^+ and $Cl^-HCO_3^-$, help to keep the internal pH at physiologic levels during resorption.¹⁶⁴ Acidification of the bone resorption compartment is dependent on osteoclast attachment to the bone surface and the formation of a seal by the clear zone.¹⁵⁹ The low pH of the bone resorption compartment leads to dissolution of bone mineral and to optimal activity of acid hydrolases.

Calmodulin, a cytoplasmic calcium-binding protein concentrated in the osteoclast cytoplasm adjacent to the ruffled border, regulates the effects of intracellular calcium and the ATP-dependent acid transport across the ruffled border.¹⁶⁵ Calmodulin antagonists inhibit acidification of the resorption compartment and bone resorption.

During bone resorption, the calcium concentration inside the osteoclast increases to a level that ultimately deactivates the osteoclast.^{166,167} A calcium sensor in the osteoclast plasma membrane is involved in regulating signaling pathways for controlling calcium channels and the release of calcium from intracellular stores.^{167,168} The patency of this channel (calcium sensor) is controlled by the level of ionized calcium in the immediate extracellular space. The elevated cytoplasmic calcium exerts an inhibitory influence on the cytoskeleton, triggering detachment of the cell from the bone matrix and loss of the ruffled border.¹⁶⁶

Inhibition of Osteoclastic Bone Resorption

Diseases caused by increased bone resorption include osteoporosis, Paget's disease, cancer-associated bone diseases, and certain chronic inflammatory conditions such as rheumatoid arthritis and periodontitis. Knowledge of osteoclastogenesis and the mechanism of action of osteoclasts has revealed numerous strategies for inhibiting bone resorption.¹⁶⁹ However only a few can be used therapeutically, principally in the treatment and prevention of osteoporosis in postmenopausal women. Estrogen replacement therapy and the use of selective estrogen receptor modulators such as tamoxifen are used to inhibit osteoclastic bone resorption in osteoporosis.

Another class of therapeutically useful compounds with potent anti-bone-resorption action is the biphosphonates. Biphosphonates interfere with the function of guanosine triphosphate (GTP)-binding proteins, thereby leading to osteoclastic apoptosis.¹⁶⁹

Calcitonin blocks bone resorption by interacting with calcitonin receptors on osteoclasts.^{170,171} Ligand binding on the basolateral surface raises the cytoplasmic calcium concentration and blocks H^+ extrusion. Calcitonin also disrupts the clear zone cytoskeleton and decreases expression of osteopontin.¹⁷² Within minutes of exposure to calcitonin, osteoclasts become detached from the bone surface and lose ruffled borders.

Osteoclastic bone resorption is decreased by the autocrine action of TGF- β . This regulator is secreted by osteoclasts, and it is liberated from bone matrix by proteolytic action of the osteoclast. Transforming growth factor β also inhibits osteoclasts by stimulating the production of osteoprotegerin by bone marrow stromal cells.¹⁷³

Additional approaches to blocking osteoclastic bone resorption include:

1. Blocking the attachment of osteoclasts to the bone surface with antibodies to integrins and/or to RGD-containing matrix proteins.
2. Blocking integrins with RGD peptides.
3. Blocking the enzyme carbonic anhydrase with acetazolamide.
4. Blocking TRAP with anti-TRAP antibodies.
5. Blocking active transport systems, including the proton pump and Na^+K^+ -ATPase.
6. Inhibiting the formation of new osteoclasts with transforming growth factor β .

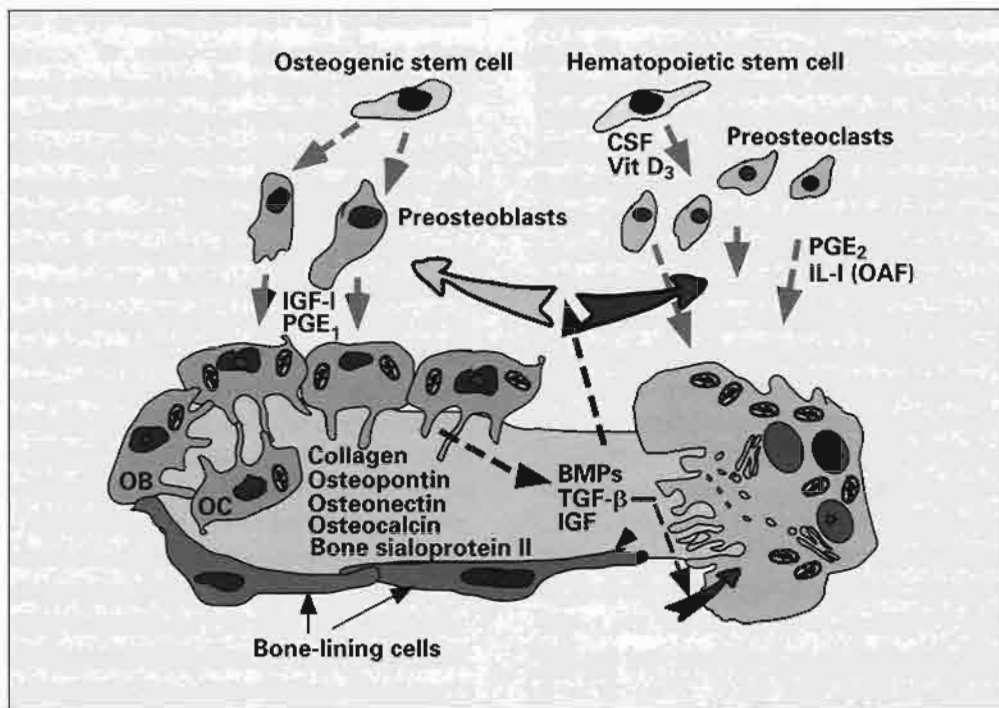


Fig 8-14 Potential central role of transforming growth factor β (TGF- β) and bone morphogenetic proteins (BMPs) in the coupling of bone formation to bone resorption. Transforming growth factor β is released from bone matrix during osteoclastic bone resorption. It acts as an autocrine factor to inhibit osteoclastic activity, and it blocks the formation of osteoclasts (OC) from precursors. Transforming growth factor β has a stimulatory effect on the differentiation of osteogenic cells and the deposition of bone matrix. Other factors act systemically and locally to modulate either the formative or the resorptive leg of the bone cycle. In general, new bone formation will occur over the bone surface vacated by the osteoclast. (green arrows) Stimulatory action; (red arrows) inhibitory action; (CSF) colony-stimulating factor; (IGF) insulin-like growth factor; (IL-1) interleukin 1 [(OAF) osteoclast-activating factor]; (PGE₁) prostaglandin E₁; (PGE₂) prostaglandin E₂; (OB) osteoblast; (Vit D₃) vitamin D₃.

7. Blocking the degradative activity of metalloproteinases.
8. Blocking the activity of cysteine proteases.

Most of these blocking strategies have been demonstrated *in vitro* and are inappropriate for clinical use either because they have widespread toxic effects or they would arrest physiologic bone resorption systemically. Clinical application in dental practice requires local inhibition of bone resorption at the site of inflammation. Nonsteroidal anti-inflammatory agents that block the actions of IL-1 and PGE₂ and chemically modified tetracyclines that block metalloproteinases have proven to reduce pathologic bone resorption in animal models.¹⁷⁴⁻¹⁷⁶ Recent attempts have been made to block bone resorption in rats with periodontal disease through injections of soluble receptors for IL-1 and TNF that inhibit the spread of inflammatory cells into the alveolar bone.¹⁷⁷

Coupling of Bone Formation and Resorption

Under normal conditions, bone formation is coupled to bone resorption in a metabolically controlled sequence of cell activations.¹⁷⁸ The sequence of cellular events is locally controlled by autoregulatory mechanisms. On a bone surface targeted for resorption, a 10-day osteoclastic resorptive phase will be followed by a repair phase that lasts about 3 months. During the repair phase, a cascade of differentiation events, including chemotaxis and cell attachment, mitosis, and differentiation of osteoblast precursors, takes place in the bone-related microenvironment (Fig 8-14). The process leads to the deposition of new bone to repair the void created by prior osteoclastic resorption. The first secretory function of the newly differ-

entiated osteoblasts is the deposition of an osteopontin-rich cement line.⁶⁴

Several growth factors and adhesion molecules have been identified as potential coupling factors between bone formation and resorption. These coupling factors are believed to act locally in autocrine and paracrine fashions on bone cells and their precursors.¹⁷⁹ Some coupling factors are produced by osteoblasts and are stored in bone matrix in association with specific binding proteins. Prime candidates for coupling agents are members of the transforming growth factor β family and insulin-like growth factor¹⁸⁰ (see Fig 8-14). Coupling factors are released from bone matrix, and from their binding proteins during bone resorption, by the acidic environment created by osteoclasts.¹⁸¹

Coupling factors inhibit further osteoclastic matrix degradation through a negative feedback loop, suppress osteoclast formation, and stimulate the proliferation and differentiation of osteoblasts and the formation of new bone (see Fig 8-14). The cycle is repeated when stimuli acting on osteogenic cells lead to osteoid degradation and the release of pre-osteoclast chemoattractants and osteoclast differentiation factors.¹⁸² The principle of bone coupling is demonstrated during the replacement of live autologous bone grafts by new bone formation.

Interleukin 1, a proinflammatory cytokine, acts as a bone uncoupler by inhibiting bone formation while promoting bone resorption. Because IL-1 is present in inflamed gingival and periodontal connective tissues, it has been proposed that the progression of alveolar bone loss in periodontitis is in part caused by disruption of coupling.

Influence of Parathyroid Hormone and Calcitonin on Bone Cells

Two endocrine hormones, PTH and calcitonin, play a major role in maintaining the blood concentration of calcium at its normal physiologic level (8.5 to 10.5 mg/dL). The chief cells of the parathyroid gland secrete PTH. It raises blood calcium by increasing the mobilization of calcium from bone, while simultaneously increasing the absorption of calcium across the gut and the reabsorption of calcium by the renal tubules. Calcitonin, produced in the parafollicular cells of the thyroid gland, acts to decrease blood calcium by decreasing bone resorption, promoting Ca^{++} excretion in the kidneys, and antagonizing the action of vitamin D in Ca^{++} uptake across the intestinal epithelium.

Effect on osteoclasts

Whether or not PTH receptors are present on mature osteoclasts is still debatable. Evidence in support of PTH receptors in osteoclasts is found in the direct and rapid generation of superoxide following exposure to the hormone.¹⁸³ However, PTH binding occurs on preosteoblasts and osteoblasts but not on osteocytes and osteoclasts.¹⁸⁴

A large body of evidence supports the view that PTH exerts many of its effects on osteoclasts indirectly by stimulating osteoblasts to release osteoclast stimulatory factors such as ODF/RANKL (see Fig 8-3).¹⁰⁹ Parathyroid hormone stimulates the osteoblastic production of IL-6, a cytokine that increases osteoclastic differentiation.¹⁸⁵ Following the administration of PTH, osteoclasts increase in size and number, and the ruffled borders and clear zones grow larger.¹⁸⁶

In contrast, calcitonin inhibits osteoclastic bone resorption by increasing cell calcium and cAMP, decreasing the size of the ruffled border, and altering the ability of the podosomes to remain attached to the bone surface.¹⁸⁷⁻¹⁹⁰ Calcitonin and its receptor are rapidly cleared from the cell membrane by receptor-mediated endocytosis. Within 15 minutes after administration of calcitonin, osteoclasts are observed to detach from bone surfaces.

Effect on osteoblasts

Parathyroid hormone stimulates the secretion of collagenase (MMP-1), gelatinase B (MMP-9), and plasminogen activator from osteoblasts. Plasmin, formed by the conversion of plasminogen, activates collagenase and other enzymes, leading to the degradation of the osteoid layer. Parathyroid hormone also causes the osteoblasts to contract, thereby uncovering some of the bone surface.³⁸ The combined effect is to make the bone surface susceptible to resorption by osteoclasts.

Paradoxically, the intermittent systemic administration of PTH increases bone formation.^{191,192} This anabolic effect is attributed mainly to the ability of PTH to increase the synthesis of IGF-I and its receptor in osteogenic cells, to stimulate cAMP and protein kinase A (PKA), and to increase gap junction communication.^{26,193}

Influence of Growth Factors and Cytokines on Bone Cells

The regulatory role of growth factors and cytokines on bone cells is highly complex.¹⁷⁹ Much of the infor-

mation available is derived from *in vitro* studies on defined osteogenic or osteoclastic cells and their precursors. The effects produced by specific agonists and/or their antagonists on bone cultures can be direct or indirect, can involve several cell types, and can result from the secondary production of additional cytokines. It is also true that hormones and growth factors have different effects, depending on the stage of differentiation of the specific target cells and their species of origin.¹⁹⁴ A given cytokine or growth factor may stimulate both osteoblasts and osteoclasts. Furthermore, a cytokine, depending on its concentration, may exert opposite effects on the same cell type.¹⁹⁵ Consequently it is often difficult to define with certainty what role a given cytokine and/or growth factor has on bone formation and resorption *in vitro* and even more so *in vivo*. Numerous interacting mediators within the local milieu undoubtedly modify the effect *in vivo* of a given factor on a cell type. The following is a summary of the actions of those agents that regulate bone cells.

Bone morphogenetic protein

Bone morphogenetic proteins and TGF- β are members of a superfamily of morphogenetic proteins that perform essential functions in embryonic development and bone cell differentiation.¹⁹⁶ Great progress has been made in isolating the members of this superfamily of morphogens, which now total about 40 proteins (see reviews by Reddi¹⁹⁷ and Sakou¹⁹⁸).

Bone morphogenetic protein 1 differs from the other BMPs in that it does not resemble TGF- β ; rather, it has been shown to be identical to procollagen C-proteinase, which processes procollagen to collagen fibrils.¹⁹⁹ Bone morphogenetic proteins 2, 3, 4, 6, and 7 have bone-inductive activity.^{200–203} Bone morphogenetic protein 2 is a chemoattractant for osteoblasts.²⁰⁴ As the structure of the BMPs was revealed, it became clear that BMP-3 was identical to osteogenin and BMP-7 to osteogenic protein 1. Bone morphogenetic protein 7 and IGF-I act synergistically to stimulate bone cell proliferation and differentiation.²⁰⁵

Bone morphogenetic proteins are expressed in bone cells as well as in a wide number of soft tissues. They were first discovered as the active ingredient of demineralized bone matrix responsible for endochondral bone induction.²⁰⁵ The expression of BMP-2, BMP-4, and BMP-7 and the presence of BMP receptors are increased in chondrogenic and osteogenic cells in sites of bone fracture repair.^{198,206} Bone morphogenetic proteins trigger increased proliferation

and differentiation of chondrogenic and osteogenic cells.¹⁹⁶ Osteoblastic cells respond to BMPs by increasing the number of PTH receptors, alkaline phosphatase activity, and the synthesis of collagen, osteocalcin, and other noncollagenous proteins.^{200,207,208} Bone morphogenetic protein 7 has been shown to activate the Cbfa1 transcription factor regulating the genes that code for bone matrix proteins.⁹

The bone-inductive actions of BMP-2 and BMP-7 have been used clinically to accelerate bone healing and to create new bone in osseous defects.^{198,209} To activate bone differentiation, BMPs are best administered immobilized in a collagenous matrix (although synthetic polymers also work as stabilizers).¹⁹⁶ In addition BMP-2 enhances the expression of IL-6 and TGF- β in osteoblastic cells.²¹⁰ Both factors may have autocrine- and paracrine-mediated regulatory effects on adjacent bone cells.

Basic fibroblast growth factor

Basic fibroblast growth factor increases the proliferation and differentiation of osteogenic cells. Systemic and local administration of bFGF enhances endosteal bone formation in experimental animals.²¹¹ Because bFGF increases the expression of TGF- β in osteogenic cells, it has been proposed that the osteogenic effect of FGF may be mediated by TGF- β . Basic FGF upregulates the expression of IL-6, a cytokine-activating factor for preosteoclasts, and promotes osteoclast formation.²¹²

Colony-stimulating factors

Colony-stimulating factors control hematopoiesis and in so doing contribute to an increase in the pool of osteoclast precursors. Monocyte colony-stimulating factor (also known as CSF-1) regulates the proliferation of monocytes and promotes preosteoclast differentiation.^{192,213} Colony-stimulating factor 1 is produced by osteoblasts and inserted in the plasma membrane and/or secreted into the bone matrix.⁵⁶ Granulocyte-macrophage colony-stimulating factor is an autocrine growth factor for osteoblastic cells.²¹⁴

Glucocorticoids

Glucocorticoids decrease bone formation and promote osteoclastic bone resorption *in vitro*.^{215–217} Prolonged exposure to increased levels of glucocorticoids leads to osteoporosis.^{218,219} Glucocorticoids depress osteoblastic activity by decreasing the expression of integrins and IGF.^{219,220} They also stimu-

late the secretion of collagenase by osteoblasts, which in turn degrades osteoid matrix, thereby releasing factors that activate osteoclastic activity.²²¹ Other studies have demonstrated that, in contrast to its catabolic effects noted earlier, glucocorticoids at physiologic levels may stimulate bone matrix synthesis and induce osteoclastic apoptosis.^{194,222,223}

Hepatocyte growth factor and macrophage-stimulating protein

Both hepatocyte growth factor and a related serum protein, macrophage-stimulating protein, activate bone resorption. Macrophage-stimulating protein induces rapid formation of osteoclast ruffled borders, redistribution of Src signaling kinases to the peripheral cytoplasm, and increased bone resorption.²²⁴

Immunoregulatory cytokines (interleukins)

The interleukins, a family of cytokines produced by many cell types but in high levels by activated lymphocytes and macrophages, regulate the differentiation of effector cells of the immune system. Many cells that do not belong to the immune system, such as fibroblasts and keratinocytes, are also capable of secreting interleukins.

In addition to their regulatory effects on cells of the immune system, the interleukins influence the activity of a wide variety of cells, including those of the skeletal system. Bone resorption observed in regions of inflammation is likely to be caused by locally produced interleukins and prostaglandins acting on the expression of OPG and ODF/RANKL, thereby altering the balance in favor of osteoclastogenesis.^{109,225}

Interleukin 1 is a potent stimulator of osteoclastic bone resorption.^{100,126,226} Activated monocytes, macrophages, T cells, neutrophils, fibroblasts, and epithelial cells produce IL-1 during inflammation. The bone-resorbing activity of IL-1 may occur indirectly through stimulation of PGE₂ production.^{227,228} The local production of PGE₂ and IL-1 in inflamed gingival and periodontal connective tissue is believed to be responsible for stimulating alveolar bone resorption.

Interleukin 2 and PTH stimulate osteoclastic activity indirectly by increasing osteoblastic expression of monocyte CSF (CSF-1) and IL-6.^{185,229}

Interleukins 6 and 11 promote bone resorption through an increase in the proliferation of preosteoclasts.^{230,231}

A recently discovered cytokine produced by bone marrow stromal cells, IL-11 appears to play a key role in the physiologic stimulation of osteoclast development.^{230,232} Antibodies to IL-11 and IL-6 block the osteoclast-promoting action of PTH, vitamin D₃, IL-1, and TNF, suggesting that they may be downstream effectors of bone resorption.²³⁰

In contrast, IL-4, IL-10, and IL-13 decrease bone resorption.^{101,233,234} Interleukin 10 inhibits bone resorption by decreasing the proliferation of preosteoclasts.¹⁰¹ It has also been reported that IL-10 and IL-8 stimulate osteoclastic activity by activating nitric oxide synthase in mature osteoclasts.²³⁵ Interleukin 4 stimulates the expression of alkaline phosphatase and collagen type I in osteoblasts.^{236,237}

Insulin-like growth factors

Insulin-like growth factors (IGF-I and IGF-II) are produced by several cell types, including fibroblasts and osteoblasts. Both factors are deposited in bone matrix, where they are stored in association with IGF-binding proteins.²³⁸ During bone resorption, IGFs are released from bone matrix and undergo disassociation from IGF-binding proteins to act in a delayed paracrine mode, along with TGF- β , to increase osteoblastic activity and new bone formation. Furthermore, it has been reported that TGF- β decreases the expression of IGF-binding protein, thereby making more IGF available.²³⁸ Insulin-like growth factors stimulate osteogenic cell proliferation and increase the synthesis of collagen, alkaline phosphatase, osteocalcin, and integrins in osteogenic cells.²³⁹ Because of the ability of IGFs to increase the proliferation and differentiation of osteogenic cells, they are regarded, along with TGF- β , as significant components of the coupling mechanism linking bone formation to prior osteoclastic bone resorption.¹⁸⁰

Insulin-like growth factors have also been shown to increase osteoclastic activity *in vitro*. Osteogenic cells mediate the osteoclast-stimulating effect of IGF.²⁴⁰ Because of this dual action, IGFs are thought to be regulators of bone remodeling.

Leptin

A small polypeptide hormone produced by fat cells, leptin has been shown to act as a potent inhibitor of bone formation. Leptin does not act directly on osteoblasts but instead exerts its effect through the central nervous system to regulate bone mass in a pathway that has yet to be defined.²⁴¹ Hormonal (sys-

temic) control of bone mass is coordinated by leptin, PTH, and the sex steroids.²⁴²

Platelet-derived growth factor

Platelet-derived growth factor (PDGF) acts as a chemotactic and mitogenic factor on osteoblastic cells. It increases the production of bone matrix proteins. Because PDGF is synthesized by osteoblasts in response to TGF- β stimulation, it could act like PGE₂ in an autocrine pathway to mediate the anabolic effects of TGF- β on bone formation.

Prostaglandins

Prostaglandins E₁, E₂, and F₂ (PGF₂) are potent stimulators of new bone formation.^{243,244} Prostaglandins E₂ and F₂ stimulate bone cell proliferation by activating phospholipase C and by increasing calcium influx through plasma membrane calcium channels.^{245,246} Increased cAMP has also been implicated in regulating osteoblastic cell proliferation in response to PGE₂.²⁴⁷ Recent animal studies indicate that prostaglandins can be administered locally to restore bone defects. In addition, PGE₁ and PGE₂ stimulate osteoblastic cells to produce VEGF, a mitogen for endothelial cells.⁴¹ The role of osteogenic cells in coordinating vascular proliferation by VEGF is one mechanism for ensuring an adequate blood supply for new bone formation.

It should be noted that PGE₂ also stimulates osteoclastic activity.²⁴⁸ Both the number and the size of osteoclasts increase under the influence of PGE₂. In vitro studies have shown that the osteoclast-stimulating effect of PGE₂ is mediated by osteoblasts. Osteoblasts stimulated by PGE₂ contract and thereby expose the bone surface to preosteoclasts.²⁴⁹

Prostaglandin E₂ also stimulates bone resorption in calvaria organ cultures. Resorption is blocked by antibodies directed against IL-1, suggesting that osteoclastic bone resorption stimulated by PGE₂ might be caused by increased production of IL-1.²²⁷ Because prostaglandins and interleukins have short half-lives (2 to 3 minutes), their effects are local and short acting.

Sex steroids

Sex steroids exert an overall anabolic effect on bones by stimulating the proliferation and differentiation of osteoblasts. They also decrease the transcription of the *IL6* gene.²⁵⁰⁻²⁵² The combination of osteoclastic bone resorption and decreased os-

teoblast proliferation, caused by low estrogen levels, is a common cause of osteoporosis in postmenopausal women.^{216,253}

Transforming growth factor α

Transforming growth factor α is closely homologous in structure and action to epidermal growth factor. It is produced by malignant cells and activated macrophages. The mitogenic effects of TGF- α and EGF on fibroblasts and osteogenic cells are exerted via the EGF receptor (tyrosine kinase mechanism).²⁵⁴ In general, TGF- α stimulates proliferation of preosteoblasts while decreasing the differentiated state. It also stimulates osteoclastic bone resorption. The production of TGF- α by cancer cells is in part responsible for the bone resorption associated with certain neoplasms.

Transforming growth factor β

Transforming growth factor β exerts an anabolic effect on osteogenic cells. It is a product of bone-forming cells that is stored in bone matrix. On its release during bone resorption, TGF- β exerts a paracrine effect to increase the proliferation of preosteoblasts (see Fig 8-14).²⁵⁵⁻²⁵⁹ Transforming growth factor β also acts as an autocrine factor to increase the synthesis of collagen, alkaline phosphatase, and osteopontin in osteoblasts. Because TGF- β increases the synthesis of PGE₂ and PDGF in osteoblastic cells, it has been suggested that the local anabolic effect of TGF- β on bone might in part be mediated by PGE₂ and PDGF. TGF- β also inhibits matrix degradation by autocrine-negative regulation of osteoclasts and by downregulation of ODF/RANKL.¹⁰⁹ Transforming growth factor β increases the expression of connexin 43 and cell-to-cell communication in osteogenic cells.²⁵

Recent evidence points to direct action of TGF- β in controlling osteogenic cell growth by activating key members of a signaling pathway involved in regulating gene transcription.²⁶⁰ The osteogenic potential of TGF- β has been demonstrated by its ability to act synergistically with BMP-7 (osteogenic protein 1) to induce ectopic bone formation when implanted along with a collagen matrix.²⁶¹

Tumor necrosis factor

Produced by many cancer cells, as well as bone cells, TNF increases osteoclastic activity, either by direct action (ODF/TNFSF-11) or by increasing the expression of IL-6. Recently, a member of the TNF re-

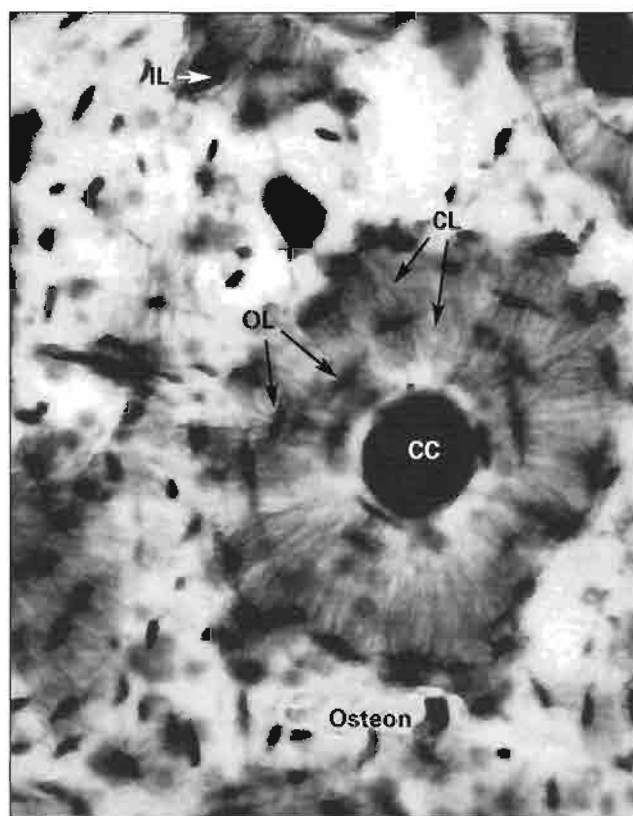


Fig 8-15 Haversian systems in compact bone. Osteocyte lacunae (OL) are arranged radially around a central canal (CC), containing nerves and blood vessels, to form an osteon. Numerous canaliculi (CL) radiate from each lacunar space. (IL) Interstitial lamellae. (Original magnification $\times 200$.)

ceptor family, osteoprotegerin, has been found to block osteoclast formation.²⁶² Osteoprotegerin has no transmembrane domain and is secreted as a soluble protein by osteoblasts in response to vitamin D and BMP-2. In contrast, the secretion of TNFSF-11 by osteoblasts in response to stimulation by PTH and IL-1 increases osteoclastic activity.

Architecture of the Bone and Replacement of Osteons

The shape and mass of individual bones are the result of external and internal bone remodeling. Both are under genetic and epigenetic control during skeletal development. External remodeling diminishes as individual bones acquire their mature form;

thereafter bone turnover in the mature skeleton occurs primarily by internal remodeling in response to mechanical and physiologic demands.

In areas of rapid bone formation, such as during embryonic development, wound repair, or at sites of high physiologic turnover (the cribriform plate of alveolar bone), the bone matrix is rapidly deposited as a coarsely arranged collagenous network, and the osteocytes are irregularly dispersed in the bone matrix. Bone of this type is described as having the appearance of a woven fibrillar matrix. In contrast, more slowly deposited bone has a more regularly arranged fibrillar matrix, and the osteocytes are entrapped in matrix in a coordinated manner. This slower and more highly coordinated activity results in a lamellar pattern in the deposited bone. The lamellar pattern of matrix deposition is the principal architectural characteristic of mature bone.

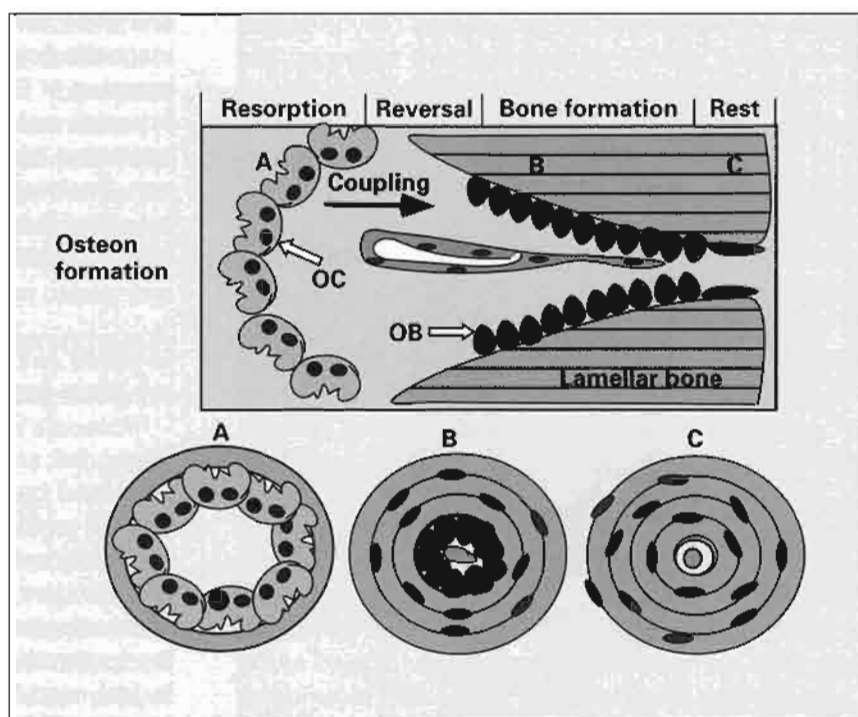
As skeletal tissue matures, woven bone is replaced by lamellar bone. This process is intimately related to the anatomy of the vascular bed. The close association of bone formation and resorption with vascular tissue is clearly demonstrated by the formation of haversian systems or osteons (Fig 8-15).²⁶³ Osteons, populated by osteogenic and osteoclastic cells, form the basic structural and physiologic units of mature compact bone.

Osteons develop by radial deposition of lamellar bone around a central core of connective tissue containing blood vessels and nerves. The maximum thickness of the osteon bony wall, about 80 μm , is limited by the ability of the outermost osteocytes to receive nourishment by cellular and extracellular diffusion of metabolites from the central canal. Osteons and haversian canals are generally oriented parallel to the long axis of a bone and are approximately 2 to 3 mm long. Volkmann's canals join the central or haversian canals laterally. They connect larger blood vessels of the periosteum to the deeper parts of compact or cortical bone.

As osteons age, the density of the bone mineral increases, and some osteocytes undergo degeneration. Micropetrosis, or excessive hardening of bone, occurs following the death of osteocytes.²⁶³ Increased mineral density of an osteon eventually leads to its being selected for replacement. The exact natures of the signals that control this process have yet to be identified. The formation of replacement (secondary) osteons occurs by the coordinated coupling of bone resorption and bone formation.

Osteon replacement begins with the development of several large osteoclasts adjacent to blood vessels in older haversian or Volkmann's canals. The osteo-

Fig 8-16 Secondary osteon formation. (A) Initiation occurs when preosteoclasts are attracted to a specific region of compact bone and fuse and differentiate into multinucleated osteoclasts (OC). This process is believed to be triggered by signals originating from local osteocytes and/or bone-lining cells. Resorption of matrix releases coupling factors (probably transforming growth factor β), which in turn stimulate osteoblastic differentiation of precursor cells (reversal). (B) Newly developed osteoblasts (OB) deposit lamellar bone. (C) After the full thickness of the osteon has been laid down, osteoblastic activity is downregulated and the osteon enters a resting phase. (Adapted from Parfitt⁴⁹ with permission from John Wiley & Sons.)



clasts tunnel through the old bone, forming a cutting cone that becomes occupied by vascular tissue and osteogenic cells (Fig 8-16).^{49,263} Coupling factors released from bone matrix by osteoclasts stimulate osteoblast differentiation and new lamellar bone formation along the inside of the tunnel. New bone is laid down at the rate of 2 to 3 μm per day, slowing down as the new osteon nears completion.²⁶³

Repair of the Bone

In the hours following a bone fracture, osteoprogenitor cells in the periosteum and endosteum, along with osteogenic stem cells of the bone marrow, divide and begin migrating toward the fracture site. A steady ionic current generated from the broken ends of the bone may serve to orient and guide migratory cells to the fracture site.²⁶⁴ The blood clot that forms between the broken ends of bone is rapidly populated by immature osteogenic cells. New blood vessels and connective tissue begin to penetrate the

clot within 24 hours. Growth factors, including BMPs, fibroblast growth factor (FGF), PDGF, and IGFs, are released, stimulating a differentiation cascade that leads to new bone formation.²⁶⁵

Within a few days, a callus of repair tissue containing new woven bone and cartilage unites the bone fragments. In the poorly vascularized parts of the callus, osteogenic cells differentiate into chondroblasts. In the next few weeks, as the callus matures and is better vascularized, cartilage is resorbed and replaced by woven bone, and after several months bone remodeling progresses to recontour the bone to its original dimension and shape.

Similar to the repair of a bone fracture, efficient healing of an extraction socket requires sufficient bleeding for the formation of a good blood clot between the walls of the socket. It is sometimes necessary to create additional openings through the cribriform plate into the adjacent marrow to stimulate bleeding and clot formation. A framework for new bone deposition is formed by type III collagen fibers that originate from the socket wall.²⁶⁶ New trabeculae

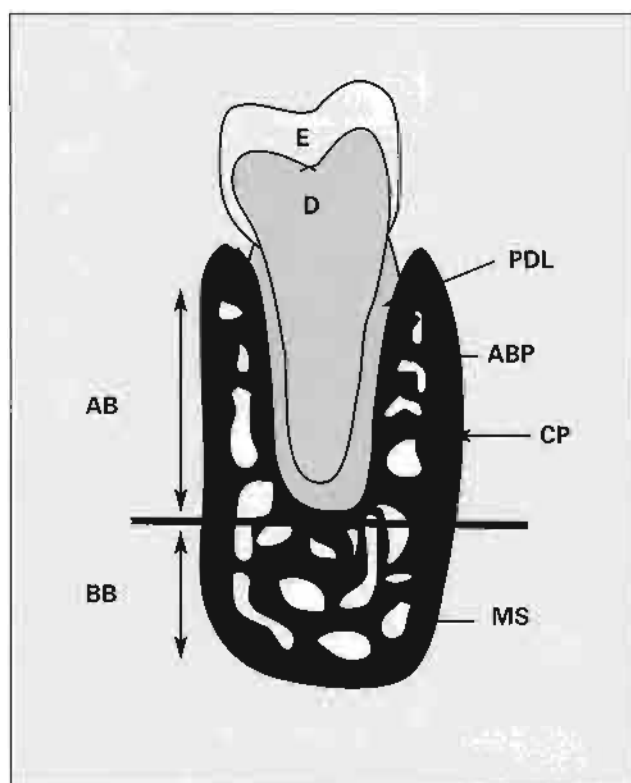


Fig 8-17 Jawbone in cross section. The outermost regions of bone, the outer and inner cortical plates (CP), consist of compact lamellar bone containing typical haversian systems. The alveolar bone proper (ABP), also known as the *cribriform plate*, is made up of bundle bone. Bundle bone contains Sharpey's fibers and abuts on the periodontal ligament (PDL). Trabeculae of lamellar bone forms an inner branching network, delimiting medullary spaces (MS) that contain hematopoietic marrow in younger individuals and a fatty and fibrotic marrow in older individuals. (AB) Alveolar bone; (BB) basal bone; (D) dentin; (E) enamel.

of woven bone develop rapidly to fill the void created by the extracted root.

New gene transfer technology may lead to improved therapeutic applications in wound repair. In vivo genetic manipulation of host fibroblasts, through the implantation of degradable matrices containing expression plasmids for BMP-4 and PTH, accelerated bone repair in experimental animals.²⁶⁷ Fibroblasts that migrate into degradable matrix incorporate the plasmids, thereby increasing growth factor synthesis.

Another method for promoting bone formation is electrical stimulation of the repair tissue. Electrical currents and electromagnetic stimuli lead to increased bone formation in cultured osteogenic cells

and in repair tissue in vivo.^{268,269} Pulsating electromagnetic fields have been shown to increase the expression of BMP-2 and BMP-4 mRNAs in chick embryonic calvaria.²⁷⁰ The electrical charge of implanted biopolymers also regulates bone formation. Positively charged surfaces appear to bind osteogenic substances that promote osteoblast and osteoclast differentiation and new bone formation.²⁷¹

Anatomic Characteristics of the Jawbones

Jawbones are functionally subdivided into alveolar bone and basal bone (Fig 8-17). The alveolar bone and the tooth it supports can be considered as a single functional unit. The development of alveolar bone is dependent on root formation. Continued presence of the tooth; after tooth extraction some alveolar bone is lost, and in edentulous individuals the alveolar bone may be resorbed down to the basal bone. Basal bone houses the major nerves and blood vessels of the jawbones and functions as a site of muscle attachment.

The external surfaces (cortical plates) of the jawbones are constructed of compact lamellar bone. Typical haversian systems are present in the cortical plates.

In contrast, the tooth-bearing surfaces of the jawbones are made up of bundle bone (also called *woven bone*). Thus the internal wall of the alveolus is constructed of coarser and less mature bone than the lamellar bone deposited in the cortical plates. This internal alveolar wall is also called the *cribriform plate* because of the many openings for blood vessels and nerves that communicate between the marrow spaces and the PDL. There are more openings in the cribriform plate toward the crestal area of the alveolus, especially in posterior teeth. On radiographs, the cribriform plate is observed as a dense border or lamina dura.

The inner compartment of the maxilla and the mandible, the medullary bone or spongiosa, is made up of trabecular bone and marrow. With increasing age, the hematopoietic elements of the marrow are replaced by adipose and fibrous tissues. Interdental and interradicular sites of alveolar bone contain spongy (trabecular) bone with trabeculae arranged along lines of functional pressure generated by mastication. In the mandible, the trabeculae are frequently arranged in a ladderlike configuration perpendicular to the long axis of the teeth.

The vestibular plate is usually thin because of the forward placement of the teeth. Root exposure is a common finding in the anterior segment. Exposures are categorized as dehiscences or fenestrations. A fenestration is an opening in the cortical plate with the crestal area intact. In a dehiscence, the defect also includes the crestal bone. Fenestrations occur in about 17% of teeth, mostly in the vestibular cortical plate. They are rarely observed in the palatal and lingual cortical plates.

An outstanding series of photographic plates illustrating the gross and microscopic anatomy of alveolar bone is contained in Schroeder's book.²⁷²

Turnover and Remodeling of Alveolar Bone

Alveolar bone turns over more rapidly than do other parts of the skeleton. The highest level of resorption and formation occurs in the cribriform plate, the layer of bundle bone adjacent to the PDL.

Part of the turnover is related to normal remodeling to accommodate the mesial and occlusal shifting of teeth over time. These shifts arise in part from unopposed tractional forces produced in the connective tissues of the periodontium and in part from mastication. Mesial drift, totaling about 3 to 4 mm during a normal life span, compensates for enamel wear at the contact points of adjacent teeth. In occlusal drift, the teeth erupt to compensate for coronal wear caused by the mastication of abrasive foods. Occlusal abnormalities created by disease or by iatrogenic means cause remodeling of the root surface and the alveolar wall.

Basic Science Correlations

Biologic mineralization of tissues

Biologic mineralization is a complex process that remains incompletely understood. Neuman and Neuman's classic monograph²⁷³ on the subject continues to be essential reading for anyone hoping to understand the fundamental aspects of biologic mineralization. The following is a highly simplified account of the currently held concepts.

Because the interstitial fluids are supersaturated with respect to bone mineral (hydroxyapatite), any discussion of biomineralization must deal with the issue of how soft tissues avoid becoming mineralized as well as explain how hard tissues undergo a

regulated and orderly mineral deposition. It is generally accepted that soft tissues contain inhibitors of crystal nucleation. These same inhibitors must be degraded at sites of hard tissue deposition in order to initiate crystal nucleation.

Because the energy required for the formation of crystal nuclei is higher than that needed for continued crystal growth, the crucial step in biologic mineralization is the formation of crystal nuclei.²⁷³ Once nuclei are established, the level of supersaturation of the interstitial fluids is high enough for the growth of hydroxyapatite crystals. The energy needed for nucleation can be met by elevating the local ionic concentration of calcium and phosphate (booster mechanism), resulting in *homogenous nucleation*.

Another way of overcoming the energy barrier is to provide substrates that can bind calcium and/or phosphate in sufficient quantity and in a topologic organization that mimics the atomic lattice distribution of the crystal phase. By concentrating the ionic participants in close and stereologically correct position, crystal nuclei are formed in a "seeding" mechanism. Because of the need for a substrate, this process is called *heterogenous nucleation*. Heterogenous nucleation is an effective way to create oriented crystal architecture as in enamel, bone, and dentin. Nature has evolved systems that utilize both homogenous and heterogenous nucleation to produce mineralized tissues.

Two biologic mechanisms are known to nucleate bone crystals. Nucleation can occur in extracellular matrix vesicles (bone, cartilage, and dentin) or in relation to collagen fibrils and associated phosphoproteins (bone and dentin). Matrix vesicles are shed from cell processes of odontoblasts, chondrocytes, and osteoblasts at sites of initial mineralization.²⁷⁴ Enzymes contained in the matrix vesicle membrane concentrate calcium and phosphate inside the vesicle above the energy level needed for nucleation.²⁷⁵ (The role of matrix vesicles in cartilage mineralization is discussed further in chapter 12.)

High-resolution electron microscopic images of the early mineralization of bone and dentin reveal that the initial mineral deposits occur in the hole regions of the collagen fibrils (Fig 8-18). The relatively small hydroxyapatite crystals of bone, dentin, and cementum grow within the hole regions, parallel to the collagen fibrils.^{276,277} Phosphoproteins and calcium-binding proteins (such as osteocalcin and/or bone sialoprotein) that bind to collagen are believed to reside in the hole regions and to be involved in the heterogenous nucleation of the crystals of hydroxyapatite (see Fig 8-18). Examination of mineralized turkey leg tendon by

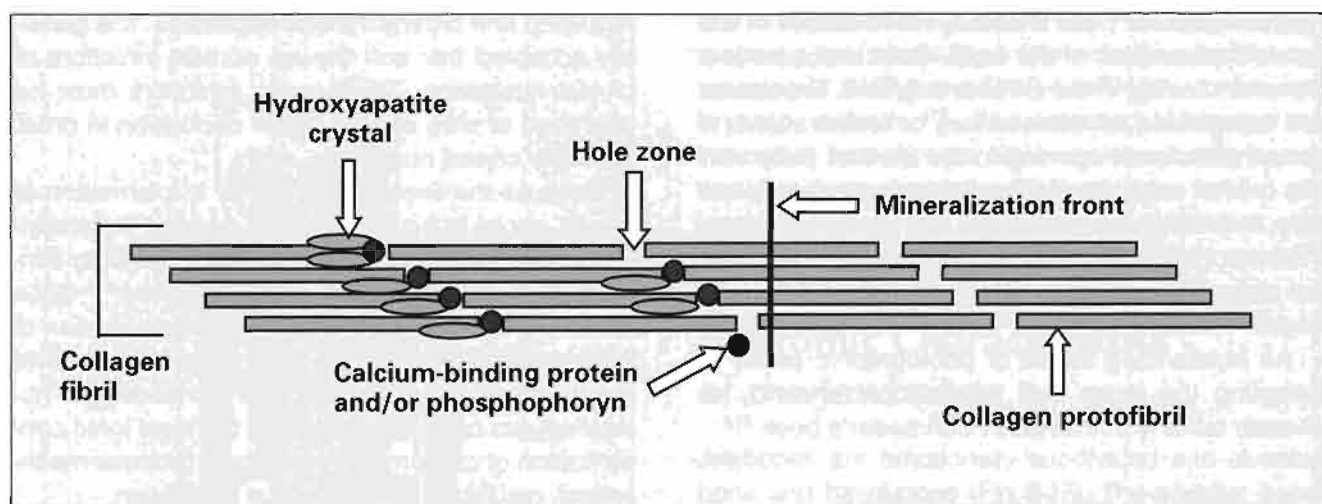


Fig 8-18 Staggered lateral association of collagen protofibrils to form a larger collagen fibril. Regularly spaced hole zones created during polymerization are believed to be potential sites for linkage of phosphophoryns and/or calcium-binding proteins. Nuclei of hydroxyapatite crystals are formed at the hole zone, and subsequently the crystals grow parallel to the long axis of the collagen protofibrils. (Adapted from Landis with permission from Elsevier Science.²⁷⁶)

atomic force microscopy has shown that a great deal of mineral is deposited in the interfibrillar spaces, suggesting that noncollagenous matrix might also have a nucleating role in bone formation.²⁷⁸ In enamel formation, the amelogenins might provide a three-dimensional scaffold, a function analogous to that provided by collagen in dentin and bone, with the important distinction that the amelogenins are degraded during the phase of crystal growth.

Active transport of calcium across the apical plasma membranes of the cells responsible for hard tissue deposition has been proposed, based on cytochemical evidence.¹⁹ Furthermore, alkaline phosphatase present at the cell surface or in the fluid phase may be responsible for locally generating phosphate groups and degrading nucleation inhibitors.

By bringing into play mechanisms that (1) degrade inhibitors of crystal nucleation, (2) boost local ion concentrations, and (3) bind ions on templates that mimic the surface of the crystal lattice, cells are able to create local environments that support an orderly and structured deposition of mineral crystals.

Gene expression and signal transduction in control of bone development

Osteoprogenitor cell proliferation and osteoblast differentiation are regulated by numerous growth factors, hormones, and cytokines operating via several cell membrane and cytosolic receptors (Fig 8-19).^{279,280} Most receptors and their corresponding

signal transduction pathways are common to many different types of cells. Tissue-specific nuclear transcription factors, the end targets of the signal transduction pathways, are responsible for initiating the expression of specific gene products of a given tissue type. Hormones and growth factors control bone cell proliferation and differentiation through the activation of nuclear transcription factors that regulate the expression of cell cycle proteins and differentiation products.

The activating protein 1 (AP1) transcription factor complex represents one of the most highly studied regulators of bone cell proliferation. The AP1 complex is formed by the association of proteins encoded by genes (*c-fos* and *c-jun*) expressed rapidly and transiently following mitogenic stimulation. Overexpression of *c-fos* induces osteosarcomas.²⁸¹ It appears that *c-fos* increases the expression of the G1 phase cyclin D, leading to uncontrolled proliferation. Under physiologic conditions, PTH increases *c-fos* transcription through the cAMP-PKA pathway and calcium-cAMP response element.²⁸² The transcription of *c-fos* is also needed for normal osteoclast formation. Decreased expression of *c-fos* has been linked to osteopetrosis, a consequence of the failure of normal bone resorption.²⁸³

A recent discovery, highly significant to bone cell differentiation, has been the identification of a "master" transcription factor that regulates the expression of genes coding for osteocalcin, osteopontin, bone sialoprotein, and collagen type I in bone cells.¹⁰ The

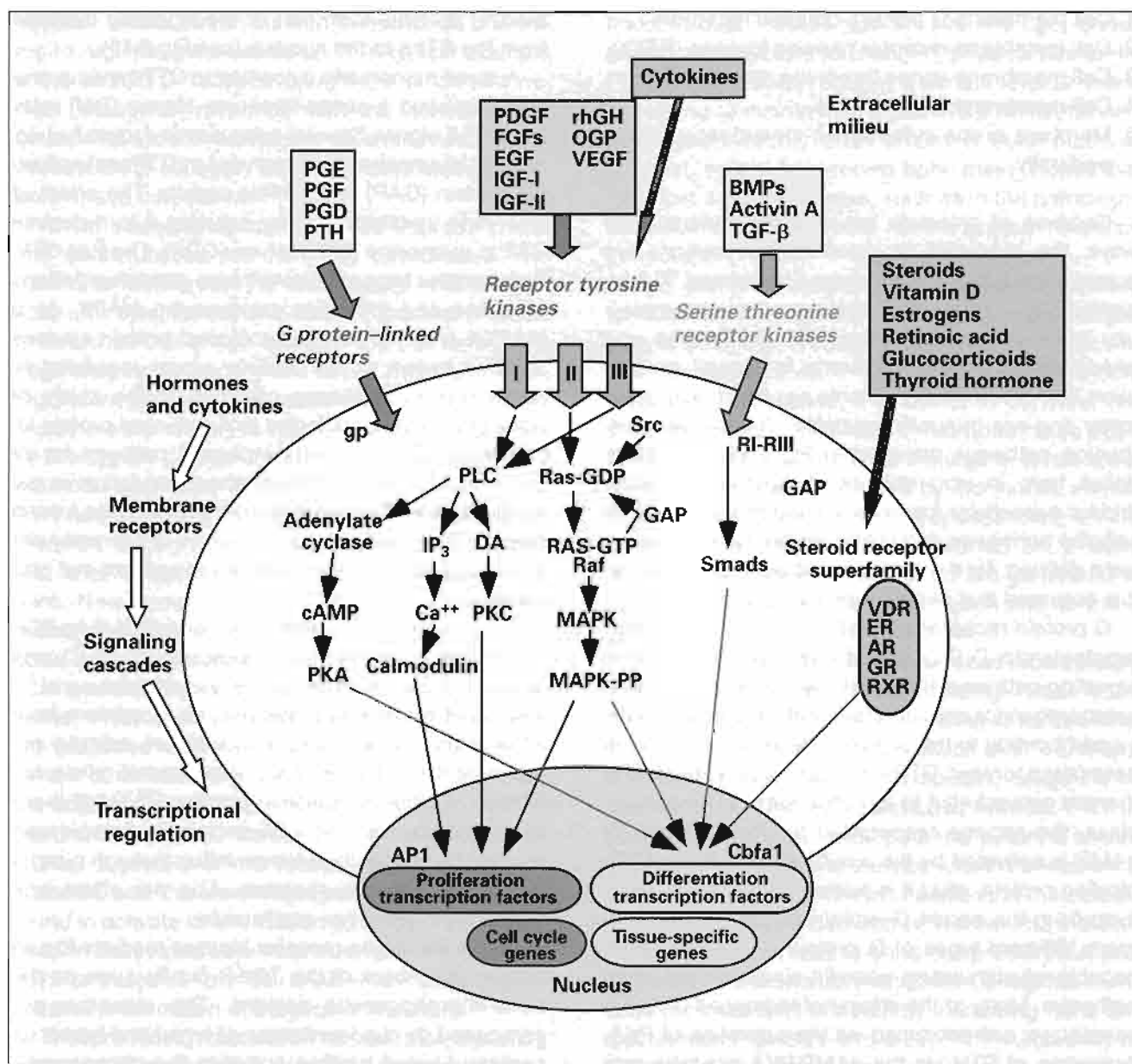


Fig 8-19 Various classes of receptors that bind hormones and growth factors at the cell membrane or, in the case of steroids, in the cytoplasm. Receptor-ligand binding triggers signaling cascades involving protein phosphorylations carried out by protein kinases. The ultimate regulatory action occurs in the nucleus, where transcription factors are activated to direct the synthesis of specific messenger ribonucleic acids (mRNAs). In the cytoplasm, the mRNA is translated on ribosomes to produce effector molecules and matrix components. Pathways are explained in the text (rhGH) Recombinant human growth hormone; (OGP) osteogenic growth peptide; (gp) trimeric guanosine triphosphate-binding protein; (RI) class I receptor tyrosine kinase; (RIII) class III receptor tyrosine kinase; (VDR) vitamin D receptor; (ER) estrogen receptor, (AR) retinoic acid receptor; (GR) glucocorticoid receptor; (RXR) thyroid hormone receptor. (Adapted from Siddhanti and Quarles with permission from John Wiley & Sons.²⁸⁰)

core-binding factor a1 (*Cbfa1*) gene is essential for bone formation. *Cbfa1* knockout mice fail to develop bone and die soon after birth from respiratory arrest. New studies have shown that in PDL fibroblasts stimulated by mechanical stretching to undergo osteoblastic differentiation, *Cbfa1* is activated by phos-

phorylation by mitogen-activated protein kinase (MAPK).²⁸⁴ Expression of *Cbfa1* protein is induced by BMP-7 and decreased by vitamin D₃.⁹

At least five receptor classes and their associated transduction pathways are believed to control proliferation and differentiation events in bone cells²⁸⁵:

1. Cell membrane G protein-coupled receptors
2. Cell membrane receptor tyrosine kinases (RTKs)
3. Cell membrane serine threonine receptor kinases
4. Cell membrane ion channels
5. Members of the cytoplasmic steroid-receptor superfamily

Because of crosstalk between the various pathways, the cascades of cytoplasmic reactions are complex and to a great degree interrelated.²⁸⁰ Integration occurring at cytoplasmic and nuclear molecular nodal points determines cell proliferation and phenotypic differentiation events. In general, proliferation and differentiation events are controlled separately and are mutually exclusive. The signal transduction pathways depicted in Fig 8-19 were elucidated from *in vitro* studies of various cell types and/or subcellular fractions. Although the effects of specific hormones and cytokines on bone formation were defined, for the most part, in bone cell cultures, it is assumed that similar events occur *in vivo*.

G protein receptor agonists, such as PGE, PGF_{2a}, prostaglandin D (PGD), and PTH, stimulate several signaling pathways that produce different effects on osteoprogenitor cells and differentiating osteoblasts. Ligand binding to the G protein receptor permits the associated trimeric GTP-binding protein to bind GTP, thereby converting it to its active form. Adenylate cyclase, the enzyme responsible for the synthesis of cAMP, is activated by the α subunit (G α) of the GTP-binding protein, after it is released in its GTP-bound form from the parent G protein receptor. There are many different types of G protein α subunits, each capable of stimulating specific signal transduction pathways. Many of the effects of increased cAMP in osteoblasts are produced via the activation of PKA. Activation of PTH via the cAMP-PKA cascade activates transcription promoters (calcium-cAMP response elements) to upregulate differentiation markers such as alkaline phosphatase, bone sialoprotein, and collagen synthesis.^{193,282,286} Stimulation of the phospholipase C-diacylglycerol-phosphokinase C pathway stimulates division of osteoprogenitor cells. This pathway appears to be preferentially stimulated by PGE.²⁴⁵ A similar response occurs via the release of Ca²⁺ from intracellular stores stimulated by inositol triphosphate (IP₃).

Activation of membrane RTKs by platelet-derived growth factor, fibroblast growth factor, epidermal growth factor, and insulin-like growth factors I and II stimulate the proliferation of osteoprogenitor cells.²⁷⁹ Cytoplasmic nonreceptor tyrosine kinases, such as members of the Src family, and monomeric GTP-

binding proteins form part of the signaling cascade from the RTKs to the nucleus (see Fig 8-19).

A small monomeric cytoplasmic GTP-binding protein (Ras) and a serine-threonine kinase (Raf) relay the RTK-II signal. Several cytoplasmic molecules, including guanosine triphosphatase (GTPase)-activating protein (GAP) control Ras activity. The effect of GAP is to inactivate Ras by inducing it to hydrolyze GTP to guanosine diphosphate (GDP). The Ras-GTP-Raf complex triggers downstream phosphorylations of serine and threonine residues on MAPK via a MAPKK enzyme. Mitogen-activated protein kinases are also known as *extracellular signal-regulated kinases* (ERKs). Evidence obtained in the study of bone cell cultures indicates that activated protein kinase C (PKC) represents a parallel pathway for increasing the level of MAPK phosphorylation in osteogenic cells.²⁸⁷ The phosphorylated protein kinase MAPK-PP enters the nucleus, where it interacts with gene-regulatory proteins needed to activate cell proliferation.

In contrast, activation of RTK-I and RTK-III by EGF and PDGF stimulates phospholipase C (PLC) production of inositol triphosphate and diacylglycerol.²⁷⁹ These pathways lead to the release of calcium from intracellular stores, the activation of calmodulin-dependent kinases, and the downstream phosphorylation of other cytoplasmic proteins.²⁵⁴ There is additional evidence that EGF increases cytoplasmic calcium by increasing calcium influx through plasma membrane calcium channels. The net effect promotes osteogenic cell proliferation.

Serine threonine receptor kinases mediate the effects of members of the TGF- β family, such as the bone morphogenetic proteins. The receptors are composed by oligomerization of type I and type II receptors. Ligand binding activates the phosphorylation of a class of cytoplasmic signaling proteins (Smad proteins) that translocate to the nucleus, initiating osteogenesis.¹⁹⁶

Osteoblasts also express receptors (purinoceptors) for extracellular nucleotides such as adenosine triphosphate. Nucleotide binding to the purinoceptor activates PLC, leading to the release of calcium from intracellular stores.²⁸⁸ It has been suggested that the elevated concentrations of extracellular nucleotides present in areas of tissue inflammation might have regulatory effects on osteoblastic activity, possibly by potentiating the action of PTH.

Vitamin D₃, thyroid hormone, retinoic acid, and steroid hormones diffuse through the cell membrane and then bind to specific receptors in the cytosol. The receptor-hormone complex is transported to the

nucleus, where it interacts with transcription promoters to regulate gene activation.²⁸⁵ A typical example is the vitamin D-receptor complex. It binds to a nuclear vitamin D response element, activating the genes that induce osteoblast differentiation, ie, the production of collagen, alkaline phosphatase, osteopontin, and osteocalcin.²⁸⁵

It has been repeatedly observed that cell shape and gene function are intimately coordinated. The mechanism whereby the extracellular matrix interacts with cytoplasmic and nuclear skeletal proteins to influence cell shape and thereby cell function is beginning to be understood at the molecular level. Significant progress was made when nuclear matrix proteins were shown to regulate DNA transcription by altering the degree of supercoiling and bending of promoter segments of DNA.²⁸⁹ It now appears that PTH and vitamin D may change bone cell gene expression through signaling pathway intermediates that alter cytoplasmic and nuclear structural proteins. New evidence indicates that activation of cell membrane stretch receptors triggers *Cbfa1* and bone cell differentiation.

In general, signaling events involving tyrosine kinases, PKC, and the calmodulin-regulated secondary cascades regulate preosteoblast proliferation.²⁸⁰ On the other hand, signaling pathways that influence the expression of differentiation products regulate mature osteoblasts. Serine threonine receptor kinases, G protein-linked receptors, the cAMP-PKA cascade, and the steroid-receptor family are usually found to activate differentiation pathways.²⁸⁰ This distinction does not always hold true, however, because PTH stimulation of the cAMP-PKA cascade increases proliferation of bone cells in culture.²⁹⁰

The preceding discussion of receptor signaling pathways in bone cells paints an oversimplified portrait of this complex field. Despite its brevity, the description may serve as a useful construct to help students navigate their entry into this highly interesting topic. Future discoveries of signal transduction events will ultimately prove to have many significant clinical applications.

Response of bone to loading forces

The shape of a bone is in part determined by the load that it must support. Through an efficient and economical use of matrix and mineral, each bone is designed to best resist the loading forces and mechanical stresses that are placed on it. This process of functional adaptation is known as Wolff's law. Throughout the life span of an individual, from child-

hood through middle age on into old age, bones adapt their structure to changing physical demands.

In general, bone requires a certain level of stress or loading to maintain its mass. Weightlessness and prolonged inactivity leads to loss of bone mass. In contrast, exercise preserves bone mass. Bones that have lost significant mass, such as in the pathologic condition of osteoporosis, have a greater tendency to fracture.

The complex cellular mechanisms responsible for functional adaptation of bones are just beginning to be deciphered through the use of molecular biologic approaches.^{282,291-295} Before cellular mechanisms are discussed, it is useful to consider the concept put forth by Frost²⁹⁶ that bone has a built-in mechanostat with several settings or levels of response, determined in part by hormonal effects (see Fig 8-20). This theory proposes that, as the amount of mechanical strain exerted on a bone rises or falls, endogenous signals are generated in the bone to stimulate bone formation and/or bone resorption.

The cellular signals are complex and incompletely understood. It is known that loading forces compress and bend bone tissue, causing local deformation or stretching of the extracellular matrix and creating fluid flow in pericellular spaces, especially in the extensive osteocytic canalicular network.^{297,298} In addition, an increase in the electrical potential is generated across the bone surface when the load on a bone is increased.²⁹⁹ These alterations of the cellular environment are sensed and converted into molecular messages that lead to bone cell proliferation and activation in a feedback mechanism designed to reduce the local level of strain by increasing bone formation (see Figs 8-21 and 8-22).^{299,300} According to Turner,³⁰¹ bone adaptation is governed by short-duration dynamic loading.

Mechanical strain in bone is measured in units of microstrain, where 1 microstrain equals 1 μm of deformation per meter of bone length. Bone that is exposed to low levels of strain will undergo resorption in excess of bone formation, resulting in decreased bone mass (Fig 8-20).^{302,303} When the strain level is raised to the physiologic range (200 to 2,500 microstrain), a homeostatic balance is created between resorption and deposition. Higher strain levels, in the range of 2,500 to 4,000 microstrain, induce a modeling sequence wherein lamellar bone formation exceeds bone resorption. At levels greater than 4,000 microstrain, a pathologic overload is created, characterized by rapid deposition of woven bone along periosteal surfaces. The overload condition is essen-

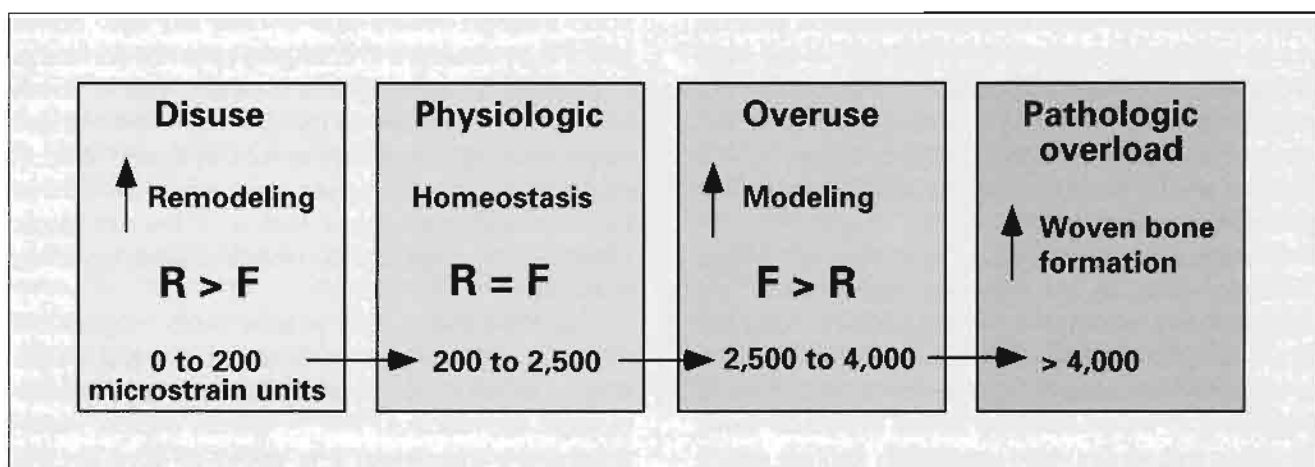


Fig 8-20 Four windows of bone activity in response to increasing loading demands. Bone remains in a homeostatic balance between resorption (R) and formation (F) at microstrain levels between 200 and 2,500. At levels of 2,500 to 4,000 microstrain, bone modeling occurs, and formation exceeds resorption. Modeling is defined as bone formation (generally lamellar bone) that alters the overall shape of a bone. At levels greater than 4,000 microstrain (overload), woven bone is deposited rapidly.

tially a repair process, similar to that involved in the healing of bone fractures.

The cellular events in the response of bone to loading forces can be divided into four phases (Figs 8-21 and 8-22)³⁰³:

1. Mechanocoupling
2. Biochemical transduction
3. Signal transmission
4. Effector cell action

The effector cells (osteoblasts and osteoclasts) are stimulated by signals arising in sensor cells (bone-lining cells and osteocytes). The concept that osteocytes and bone-lining cells are responsible for sensing changes in local strain is based on the fact that, at any one time, the total bone surface covered by effector cells is less than 10%. Furthermore, there is direct evidence that osteocytes respond to mechanical stimulation by the expression of IGF-I and by the subsequent synthesis of collagen type I and osteocalcin.³⁰⁴ Osteocytes are considered to be especially well suited to act as sensor cells because of their very high numbers, their extensive system of cell processes that ramify throughout the bone, and their ability to form gap junction communication with cells at the surface of bone.^{297,298} It has been proposed that gap junctional communication between osteocytes is an essential component in determining the net response to a given degree of loading.³⁰⁵ The functional response of the bone-lining cells and osteoblasts to mechanical strain is triggered by signals transmitted either through os-

teocyte cell processes and gap junctions or by mediators diffusing through the bone fluid.^{299,303}

Mechanotransduction in bone cells occurs through deformation of bone matrix (substrate strain) and through the effect of fluid flow (shearing force) on plasma membrane proteins.^{295,298} Osteocytes have been shown to be more sensitive to fluid shear stress than preosteal fibroblasts and osteoblasts.²⁹⁷

Although the actual events of mechanotransduction are not firmly established, there is growing evidence that mechanotransduction involves an integrated response of matrix proteins, integrins, focal adhesion kinases, and plasma membrane calcium channels (see Fig 8-21).^{297,306-309} In vitro experiments have shown that mechanical stimulation of single osteoblastic cells cause a rise in intracellular calcium and the spread, within seconds, of a calcium wave to adjacent cells.³¹⁰ The time course of the response suggests that second messengers regulate the permeability of the calcium channels. Direct stimulation of stretch-activated calcium channels is also a possibility.²⁹⁷ Calcium wave propagation in osteoblastic cells occurs through gap junctions of contiguous cells or through ATP activation of purinoceptors in neighboring cells.³¹⁰ In vitro studies have also shown that fluid flow across the surface of individual bone cells creates a shearing force that leads to an IP_3 -mediated release of calcium from cytoplasmic calcium stores.³¹¹ A similar response occurs when bone cells are exposed to increased hydrostatic pressure. Osteoblasts and osteocytes exposed to fluid flow-induced shear stress

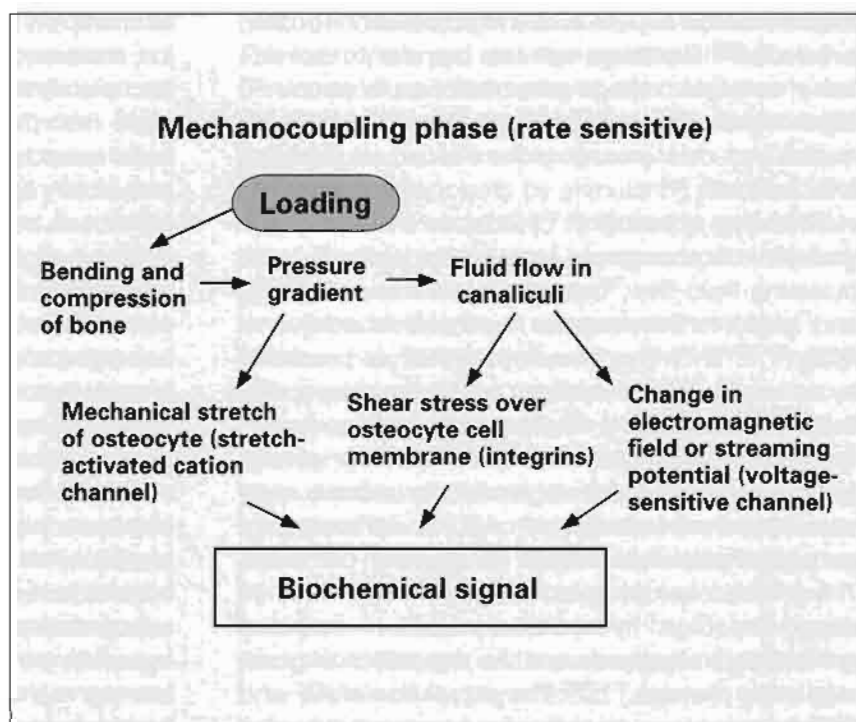


Fig 8-21 Potential mechanisms whereby deformation of bone matrix and fluid flow might interact at the cell surface to initiate a biochemical signal in a bone cell (presumably an osteocyte).

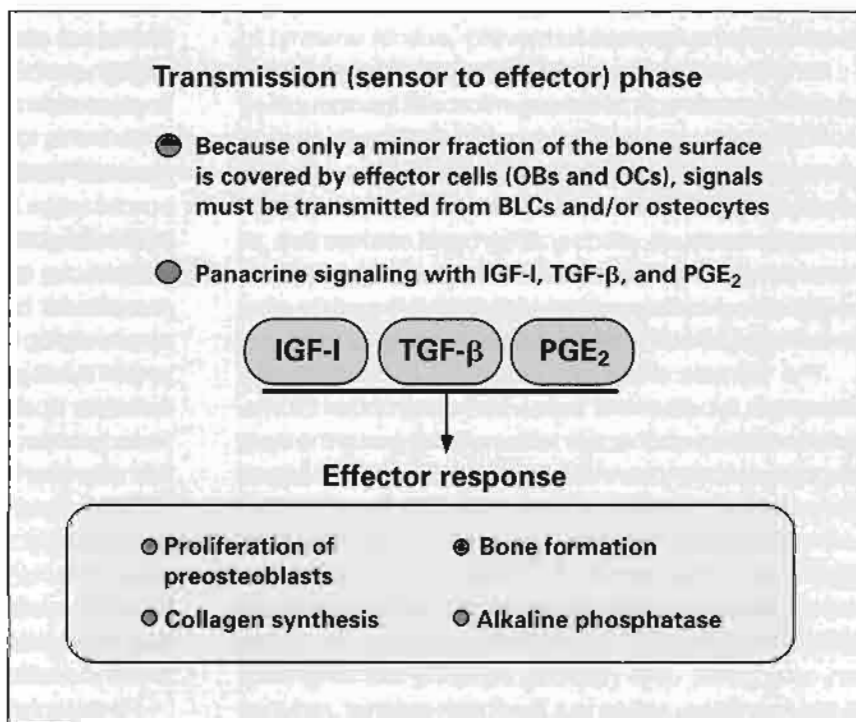


Fig 8-22 Potential mechanism for converting biochemical signal into a bone formation response. (BLCs) Bone-lining cells; (IGF-I) insulin-like growth factor I; (OBs) osteoblasts; (OCs) osteoclasts; (PGE₂) prostaglandin E₂; (TGF- β) transforming growth factor β .

produce nitric oxide, a potent bone cell mitogen and inhibitor of osteoclasts.^{312,313}

Cyclical deformation (dynamic loading) of osteogenic cells in vitro leads to cell proliferation and increased synthesis of matrix proteins and integrin

receptors.^{293,309,314} Cultures of chick calvaria osteoblasts exposed to cyclical deformation showed increased expression of osteopontin. Essential components of the transduction system include an intact microfilament system, tyrosine phosphorylation of

focal adhesion kinase, and the activation of protein kinase A.³⁰⁹ Electrical currents appear to activate bone cells via voltage-gated calcium channels.³⁰⁷ Signaling events involved in the response to deformation include increases in the second messengers cAMP and IP_3 .³¹⁵

Following exposure to cyclical deformation (compression), to changes in local electrical fields, or to pulsating fluid flow, osteogenic cells release PGE_2 and begin to increase the synthesis of additional PGE_2 .^{297,307,316,317} The elevation of PGE_2 is preceded by an increase in intracellular calcium and by activation of phospholipase A₂, an enzyme that generates arachidonic acid from membrane lipids for prostaglandin synthesis. Prostaglandin E_2 subsequently stimulates the production of cAMP and thereby triggers cAMP-dependent signal transduction pathways.

Increased compression of osteoblastic cells induced by high hydrostatic pressure increases prostaglandin synthesis and the expression of gene-regulatory proteins.³¹⁸⁻³²⁰ The expression of IGF and TGF- β by osteogenic cells also increases following exposure to loading conditions.^{304,321,322} Both factors increase bone formation.

Stretch-activated cation channels present in the plasma membrane of osteogenic cells increase their conductance of calcium ions after cell cultures are exposed to cyclical deformation. Fluid flow also generates small local electrical potentials (streaming potentials) because of the differential movement of ions. It has been suggested that streaming potentials might affect voltage-gated ion channels and/or alter the charge of cell surface proteoglycans.

The ultimate effect of local tissue and cellular deformation, whether it is sensed as a disruption of integrin-mediated anchorage or as a change in the conductivity of ion channels, is the activation of signal transduction pathways involving cytoplasmic calcium concentrations, cAMP levels, IP_3 , diacylglycerol (DAG), and PKC activity.³¹⁵ These events lead to the subsequent formation of paracrine factors, such as IGF-I, PGE_2 , and TGF- β which have an anabolic effect on osteogenic cells (see Fig 8-22).^{291,293,297,313,322} New bone formation, acting in a feedback manner, reduces the local strain imposed by the original loading force.

Clinical Correlations

Alveolar bone regeneration

The destruction of alveolar bone in periodontal disease leads to tooth loss. A primary goal of periodon-

tal therapy is to eliminate bacterial plaque and calculus, the sources of bone-resorptive stimuli. Once the bacterial challenge has been minimized and the bone resorption and connective tissue destruction have been brought under control, the tissues are poised for regenerative growth. Significant advances have been made in recent years to identify the conditions that must be created to allow the regenerative process to proceed. As discussed in chapter 6, the use of membranes to exclude unwanted cell types from the zone of regeneration and the application of growth factors to stimulate connective tissue reattachment have shown promising results in experimental animals.

Connective tissue reattachment without new alveolar bone formation is less than satisfactory for long-term survival of teeth. It is for this reason that the bone-inductive potential of BMPs has been viewed as a promising therapeutic agent in the clinical management of periodontal bony defects. Although many growth factors (IGF, TGF- β , and FGF) stimulate bone formation by increasing the proliferation of cells that are already committed to osteogenic activity, BMPs are able to induce pluripotent nonosteogenic cells in soft tissue to enter the osteogenic pathway. Implantation of BMPs in sites that do not ordinarily form bone leads to the initiation of a differentiation cascade that culminates in new bone formation. The combination of TGF- β and BMP-7 acts synergistically to increase the amount of new bone.²⁰⁹

The use of BMPs in the repair of experimental mandibular and alveolar bone defects has produced encouraging results.^{323,324} A combined approach, in which synthetic membranes are used to create and maintain space for bone regeneration, and recombinant human BMP-2 is introduced to the blood clot that fills that space, has led to significant new alveolar bone formation.³²³ Regeneration of bone in experimentally produced transosseous mandibular bone defects is greatly accelerated by locally applied BMP-2 and the use of polytetrafluoroethylene membranes to limit the new bone to the original contour of the mandible.

Periodontal and maxillofacial surgical procedures involving the use of several growth factors, including BMPs, to regenerate bone destroyed by chronic inflammation and cancer are certain to become routine.

Orthodontic tooth movement

Orthodontic tooth movement is achieved by exerting mechanical stress on the tooth to be moved with the use of various intraoral orthodontic appliances. The

stress is transmitted through the root to the periodontal tissue and alveolar bone. Compression occurs along the leading root surface, and tension occurs in the opposite or trailing side.

Excessive force must be avoided, because it creates tissue damage, necrosis, and root resorption. Light forces, however, lead to controlled tissue disruption and mild hyalinization of the PDL on the compression side. In the optimal range of force application, the cells in the compression side initiate an inflammatory-like response, similar in so far as several proinflammatory mediators are increased locally.

A rat model of orthodontic tooth movement revealed that IL-1 increased in compressed tissue and osteoclasts were recruited to compressed bone surfaces during the first day following appliance activation.³²⁵ The initial phase of osteoclast development was followed by a refractory period, when appliance reactivation failed to recruit more osteoclasts to the bone surface. However, if several days were allowed to elapse before appliance reactivation, a second wave of osteoclasts developed on the compressed bone surfaces.³²⁵

During the initial activation, osteoclasts form from precursors already positioned in the periodontal ligament.³²⁶ Because orthodontic tooth movement requires repeated reactivation over several months, preosteoclasts must be recruited into the PDL from bloodborne precursors.

During the inflammatory response, cells of the PDL and alveolar bone release cytokines and prostaglandins. Gingival crevicular fluid collected from teeth that are undergoing active orthodontic tooth movement contains increased levels of IL-1, IL-6, TNF- α , and EGF.^{327,328} These factors are capable of stimulating PDL prostaglandin synthesis and local osteoclast differentiation and bone resorption.²⁴⁸ Prostaglandin E is also expressed within PDL fibroblasts in the compression side. Specific inhibitors of prostaglandin synthesis, such as indomethacin, have been shown to reduce osteoclast recruitment and the rate of orthodontic tooth movement.^{329,330} In contrast, gingival injections of prostaglandins accelerate tooth movement.^{331,332}

Bone resorption in periodontal disease and periapical lesions

Bone resorption is the hallmark of chronic periodontal disease. Bacterial plaque is the source of various biologically active substances that induce a local inflammatory response in the soft tissues of the gin-

giva, periodontal ligament, and periapical tissues. The neutrophils and macrophages that infiltrate these tissues produce interleukins and prostaglandins that have the potential of inducing osteoclastic activity.³³³⁻³³⁵ Bacterial endotoxins are also capable of stimulating osteoclasts by stimulating the local production of IL-6 and IL-8.³³⁶ Endotoxins also activate CD4⁺ T cells that stimulate bone resorption via their interaction with macrophages.³³⁷

A particularly potent osteoclast-stimulating protein has been isolated from the fimbria of *Porphyromonas gingivalis*, an organism isolated from active human periodontal disease sites. It causes periodontal disease when introduced in gnotobiotic animals. Antibodies directed to the fimbrial protein administered to the gnotobiotically infected animals prevented periodontal tissue destruction. Inactivation of the bacterial gene responsible for coding fimbrial protein also protects against osteoclastic bone resorption. Recent evidence suggests that the fimbrial protein of *Porphyromonas gingivalis* stimulates osteoclasts via a tyrosine kinase mechanism. Blocking tyrosine kinase with genistein, a potent inhibitor of tyrosine kinase, prevented fimbrial protein-stimulated bone resorption in vitro.

Actinobacillus actinomycetemcomitans, another oral microorganism that has been implicated in the pathogenesis of localized juvenile periodontitis, produces a 62-kDa heat-shock protein associated with its cell surface that has the ability to stimulate bone resorption at picomolar concentrations.³³⁸ *Actinobacillus actinomycetemcomitans* also secretes a peptide that acts as a potent inducer of IL-6 in fibroblasts and monocytes.³³⁹

As dental scientists learn more about the pathways involved in osteoclast development and activation, and as they identify additional osteoclast-stimulating factors of oral bacterial origin, an effective local therapy to block osteoclastic activity may become a standard method of disease prevention. Nonsteroidal anti-inflammatory drugs and soluble receptors (antagonists) for IL-1 and TNF are among the strategies that show promise.¹⁷⁵⁻¹⁷⁶

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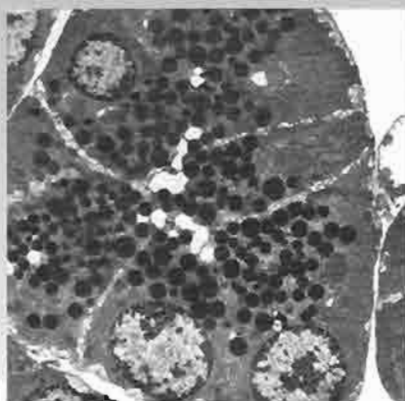
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Chapter 9

Salivary Glands



Saliva is an essential factor in the health of the oral cavity. Saliva performs the following protective and physiologic functions¹:

1. Cleansing and lubrication of oral mucosal surfaces
2. Buffering of acids through its content of bicarbonate ions
3. Antimicrobial protection, provided by secretory immunoglobulin A (IgA) antibodies and its lactoperoxidase system
4. Protection of the enamel surface, provided by negatively charged proteins that bind to hydroxyapatite
5. Initiation of the digestion of complex carbohydrates through the action of the enzyme amylase
6. Solubilization of food for the proper functioning of the taste buds

In recent years, various growth factors have been identified in saliva.² The potential role of these factors in the rapid healing of oral mucosal wounds is a topic of considerable interest and investigation.³ A partial listing of the most important components of saliva is contained in Table 9-1.

In humans, 90% of saliva is produced in the major salivary glands (the parotid, submandibular, lingual, and sublingual glands). Hundreds of minor glands lo-

cated in the submucosa throughout most of the oral cavity account for the remaining 10%.⁴

Approximately 0.5 L of saliva are secreted during a 24-hour period, most of it during the day.⁵ The flow of saliva is stimulated in part via reflex neural pathways stimulated by the actions of tasting and chewing food.⁵ Because these and other tactile stimuli are reduced during sleep, the protective functions of saliva are mainly absent when an individual is asleep.⁵ A clinical correlation of this fact is bottle-baby caries, a condition of rampant dental decay, which develops in children who go to sleep while nursing on a bottle of infant formula or juice. In this situation, a relatively constant supply of substrate for bacterial acid production is present during a period of decreased acid buffering by saliva.

General Composition of a Gland

The classic literature (here defined as preceding the development of molecular biology) dealing with the morphology and physiology of salivary glands has been compiled and synthesized into a valuable monograph by Young and van Lennep.⁶ For more recent reviews of the mechanisms of salivary function, several excellent books are also available.⁷⁻⁹

Table 9-1 Biologically important substances in saliva

Category	Substance and functions
Antibacterials	Lysozyme: Binds and degrades bacterial membranes. Lactoferrin (iron-binding protein): Deprives oral bacteria of ferric ion, an essential nutrient. Lactoperoxidase (enzyme): Uses hydrogen peroxide to produce oxidizing agents that disrupt vital bacterial enzyme systems.
Antifungals	Histidine-rich proteins (histatins): Inhibit growth of the fungus <i>Candida albicans</i> .
Antivirals	Secretory antibodies: Act against viral pathogens.
Lubricants	Mucins: Coat oral tissues, acting as a lubricant as well as a barrier to toxins.
Remineralization agents	Calcium phosphate salts, statherin, and proline-rich proteins: Inhibit the precipitation of salts in saliva so that they remain in solution, available for remineralization of the tooth surface.
Anticarcinogens	Proline-rich proteins bind tannin-rich foods.
Digestive enzymes	Amylase.
Proteases	Kallikrein: Converts kininogen to kinin; acts as a vasodilator.
Antiproteases	Cystatins (salivary phosphoproteins rich in cystine): Prevent oral tissue destruction resulting from proteases produced by bacteria in dental plaque.
Growth factors	Epidermal growth factor, nerve growth factor, mesodermal growth factor, and hepatocyte growth factor.

Salivary glands consist of multiple secretory units connected to the oral cavity by a system of ducts.⁶ Each secretory unit is a cluster of cells organized in an acinar (round cluster) or tubular (elongated cluster) configuration.¹⁰ Secretory endpieces and their associated ductal segments are organized into lobules¹¹ (Figs 9-1 and 9-2). Each gland comprises many lobules drained by second-order excretory ducts that empty into the main excretory duct.

Salivary secretory cells may be classified into two broad categories, serous-secreting and mucous-secreting cells.⁶ Serous cells produce a product that is almost entirely protein, while the mucous cells produce a product that contains only a small amount of protein but a high content of complex carbohydrates. In recent years, as new histochemical techniques have been applied to the study of salivary glands, many of the cells previously classified as serous cells have been shown to contain significant amounts of carbohydrate.⁶ These cells have been reclassified as part of a third category,

the seromucous cells.^{6,12} Because seromucous cells have the same general shape as serous cells, and both serous and seromucous cells are typically organized into acinar secretory endpieces, the terms are considered synonymous in this discussion.

A great diversity of glandular structure and granular content exists in nature.^{6,10} This reflects the adaptation of salivary fluid to diverse functions. There are significant differences between the parotid glands of carnivores and those of herbivores, in those of vampire bats and fruit-eating bats, and in those of aquatic and land mammals. Birds produce copious amounts of viscous saliva that is used in nest building. In poisonous snakes, the salivary glands have been adapted to produce venom. Sea snakes use salivary glands for the secretion of salt. All of these special physiologic adaptations are reflected in varied patterns of microanatomic structure and neural integration.

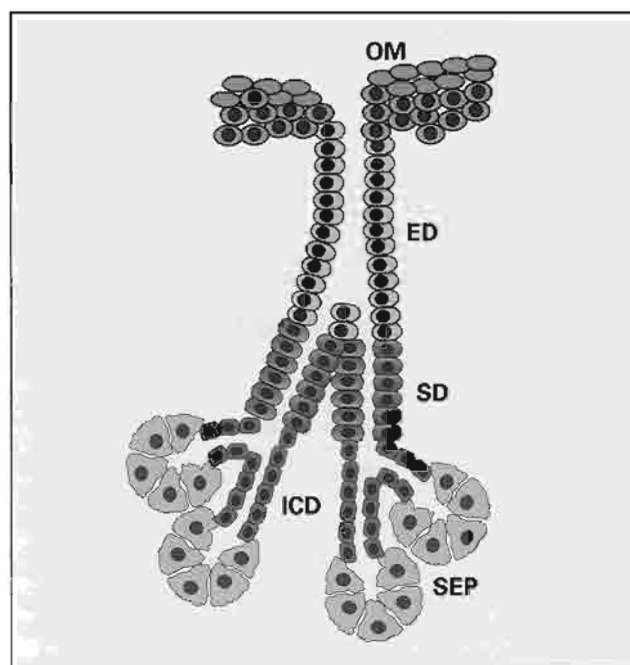


Fig 9-1 Basic composition of a salivary gland. Multiple secretory endpieces (SEP) are connected to the oral cavity through a system of branching ducts consisting of intercalated ducts (ICD), striated ducts (SD), and a major excretory duct (ED) that merges with the oral mucosa (OM).

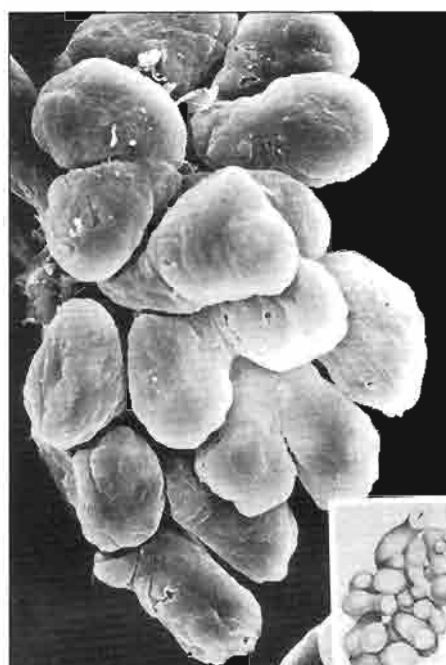


Fig 9-2 Scanning electron micrograph of an isolated salivary gland lobule composed of numerous grapelike secretory endpieces. The connective tissue was previously removed by enzymatic digestion. The inset depicts a lobule as visualized in an early anatomic drawing. (Reprinted from Riva et al¹¹ with permission from John Wiley & Sons.)

Development of the Salivary Glands

The epithelial components of the salivary glands are derived from the primitive oral epithelium. The first sign of glandular development is a thickening of the oral epithelium adjacent to a condensation of mesenchymal cells. The thickened region of the epithelium undergoes increased proliferation and invades the underlying mesenchyme in the form of a solid epithelial cord (Fig 9-3 [A and B]). This primary cord, destined to be the main excretory duct, is four cells across and is without a lumen.

The bulbous distal (growing) end of the primary cord branches to give rise to secondary epithelial cords. Branching requires coordination of cytoskeletal functions and extracellular matrix deposition and resorption. Transfilter cocultures of salivary epithelium and mesenchyme have provided evidence that soluble mesenchymal factors are essen-

tial to salivary epithelial differentiation. Early experiments with in vitro organ cultures demonstrating that collagenase stopped the branching process pointed to the importance of connective tissue matrix in epithelial-mesenchymal developmental interactions.¹³

The epithelial cells in the bulbous cell mass express the E cadherins and β -catenins of cell-to-cell adherens junctions but fail to maintain desmosomes or tight junctional contacts during the predifferentiation stage.¹⁴ While in this state, the epithelial cells are probably more plastic and susceptible to forces generated in the adjacent mesenchyme. The desmosomal and zonula occludens gene products are reexpressed as the innermost cells begin to polarize during lumenization.¹⁴

By repeated branching and continued growth, the epithelial parenchyma of the gland takes shape (Figs 9-3 [C] and 9-4). The branching process requires the presence of a basement membrane.^{15,16} Salivary epithelial cell contact to matrix molecules via integrins

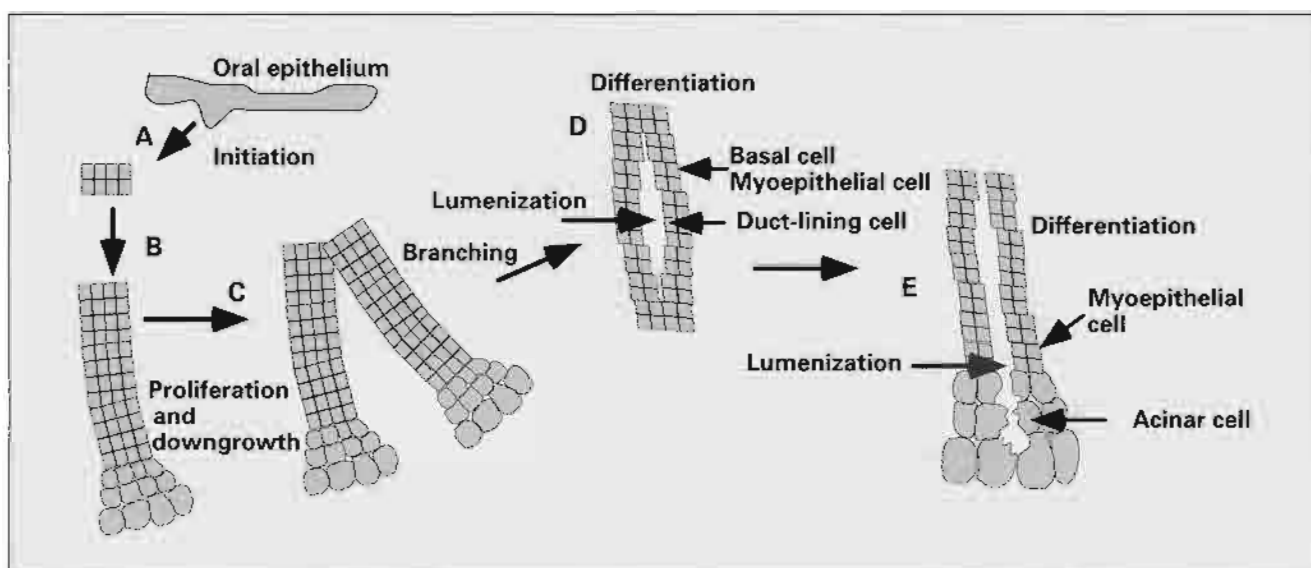


Fig 9-3 Sequential steps of epithelial growth and differentiation during salivary gland organogenesis. Initiation (A) occurs at the epithelial surface of the developing oral cavity. Proliferation, downgrowth, and branching (B and C) increase the mass of the glandular parenchyma prior to epithelial cell specialization and lumenization (D and E).

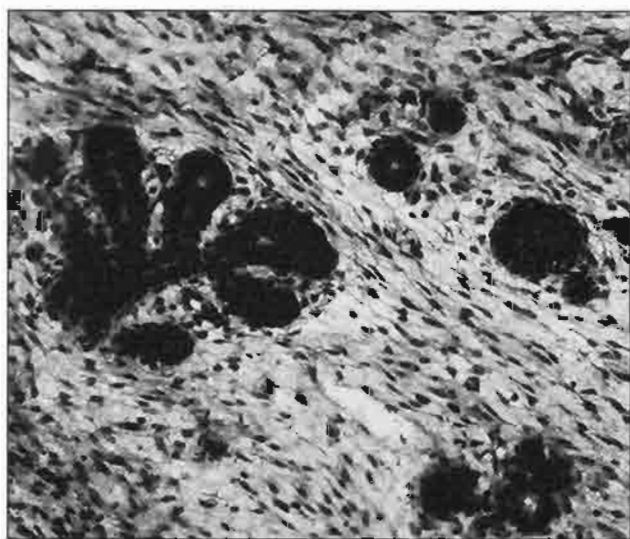


Fig 9-4 Histologic section of a developing salivary gland, illustrating budding epithelial cords at the start of lumenization and cell differentiation. (Hematoxylin-eosin stain. Original magnification $\times 120$.)

activates signaling pathways and gene expression during salivary gland development.¹⁷ Interaction between laminin and cell membrane syndecan and $\beta 1$ integrins is involved in the induction of acinar cell differentiation.^{18,19} Epidermal growth factor and its receptor appear to regulate the branching process, while fibroblast growth factor 7 (keratinocyte growth factor) controls stalk elongation.^{20,21} When the tyrosine kinase activity of epidermal growth factor receptor is blocked, branching is decreased and glandular development is arrested. It has been suggested that epidermal growth factor may control branching by

regulating the expression of $\alpha 6$ integrin laminin receptors.²⁰

The proximal end of the main epithelial cord, the end nearest the oral cavity, differentiates into the main excretory duct. Several of the first branches to form develop into second-order excretory ducts. Further branches give rise to intralobular ducts that differentiate into striated, granular, and intercalated ducts.

Lumenization of epithelial cords, beginning in the middle-to-proximal parts of the system, occurs simultaneously with differentiation of specific cell types (see Figs 9-3 [D] and 9-4). Specialization of the

cells of the inner layers, involving the establishment of cytoplasmic polarity and the development of apical junctional complexes, leads to the formation of a central extracellular space, or lumen. Cells of the inner layer differentiate into specialized cells that characterize the intercalated, granular, striated, and excretory ducts. Cells in the outer layers of the developing ductal segments differentiate into myoepithelial cells (intercalated segments), basal cells (striated and perhaps granular segments), and basal and suprabasal cells (stratified columnar and stratified squamous segments).

Mucous and serous cells differentiate from the inner-layer cells in the terminal bulbous segments of the epithelial cords. Secretory endpiece lumenization and secretory cell specialization occur after the ductal elements have established a continuous lumen communicating with the oral cavity (see Fig 9-3 [E]). The outer-layer cells of the bulbous terminal segments differentiate into myoepithelial cells.

Immunocytochemical studies of salivary glands have shown that different secretory proteins are expressed in fetal, postnatal, and adult stages of development.^{22,23} Some changes in protein expression coincide with weaning and the consumption of solid foods.^{24,25}

Axonal growth parallels the epithelial branching process during salivary gland development.²⁶ In vitro organ cultures of salivary gland epithelium and submandibular ganglia have demonstrated that axonal outgrowth from the ganglia is directed by the salivary gland epithelium.²⁷

Organogenesis and cytodifferentiation of the salivary gland have been studied almost exclusively in rat and mouse embryos. Much less is known about salivary gland development in humans. The development of the human parotid gland is initiated at the corner of the mouth from two sites of epithelial downgrowth that merge to form a single gland. Epithelial invagination begins between the 6th and 7th weeks of fetal life. The submandibular gland develops around week 6, arising from endoderm covering the floor of the mouth. Branching in the parotid and submandibular anlage begins at 8 weeks, and lumenization starts at around the 16th week in utero. Cytodifferentiation is completed about the 6th month in utero for the parotid and submandibular glands.

The sublingual gland develops on a slightly later time frame. It is initiated by downgrowth of endoderm over the paralingual sulcus at the 8th week of embryonic development. In the embryo, the minor glands begin to develop in week 12.

Basic Structure of Serous and Mucous Cells

Serous cells

The following are the characteristic features of a serous cell, as observed by light and electron microscopy^{6,28-32} (Figs 9-5 to 9-7):

1. A large, round nucleus positioned in the center of the cell
2. Numerous secretory granules in the apical or supranuclear cytoplasm
3. A basophilic infranuclear zone occupied by rough endoplasmic reticulum (RER)
4. A well-developed Golgi apparatus located just apical to the nucleus, sometimes obscured by the large number of secretory granules
5. Indistinct lateral borders, caused by the interdigitation of microvilli in the lateral intercellular spaces

Secretion granules are stained deep purple with hematoxylin and dark blue with toluidine blue. Based on ultrastructural appearance, it has been suggested that serous cells may contain more than a single class of secretory granules.

Additional features observed by electron microscopy are a large number of cytoplasmic infoldings along the basal surface abutting the basal lamina and a junctional complex consisting of a zonula occludens and a zonula adherens.^{11,30,31,33} The basal cytoplasmic infoldings interdigitate with those of adjacent cells.^{6,31} Desmosomes and gap junctions are also observed to connect adjacent cells.³⁴

The lateral intercellular spaces located apical to the zonula occludens (toward the lumen) form secretory canaliculi.^{6,30} These narrow clefts or channels are lined by microvilli and form part of the luminal membrane of the secretory endpiece.^{6,11,30,31} Quantitative analysis of the surface area of the luminal (apical) plasma membrane indicated that it is roughly a 12th of that of the basolateral membrane.³⁵ In histologic sections, the lumen appears small because the secretory canaliculi are not visible. On stimulation, the lumen increases as granule membrane is added to the apical surface.³⁶ In glands that produce a high volume of fluid, the basal and lateral microvilli are prominent.

Because of the large amount of RER needed to produce copious quantities of exportable protein, the basal cytoplasm of serous cells is amplified, giv-

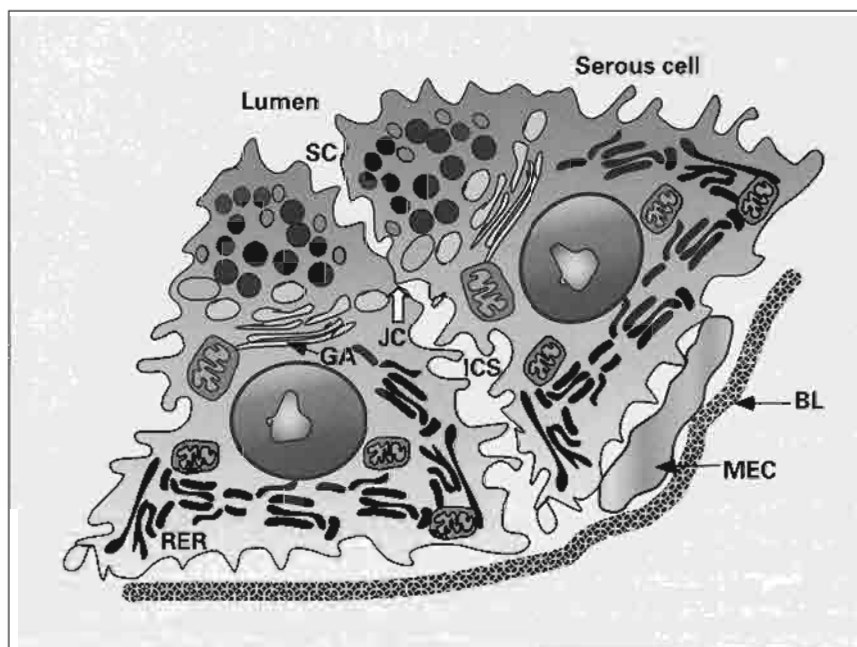


Fig 9-5 Structure of serous cells. A serous cell is pyramidal in outline, with a narrow apical surface and a wide basolateral surface in contact with the basal lamina (BL). Consistent with a high level of protein synthesis, the rough endoplasmic reticulum (RER) is well developed, occupying most of the basal and lateral cytoplasm. A well-developed Golgi apparatus (GA) is located apical to the nucleus. The nucleus is large, round, and centrally located. The apical cytoplasm is crowded by densely stained secretion granules. Secretory canaliculi (SC) form extensions of the lumen between the apical portions of the serous cells. The secretory canaliculi are separated from the intercellular space (ICS) by the junctional complex (JC), consisting of a zonula adherens and a zonula occludens. Cytoplasmic processes of myoepithelial cells (MEC) are present between the serous cell and the basal lamina.

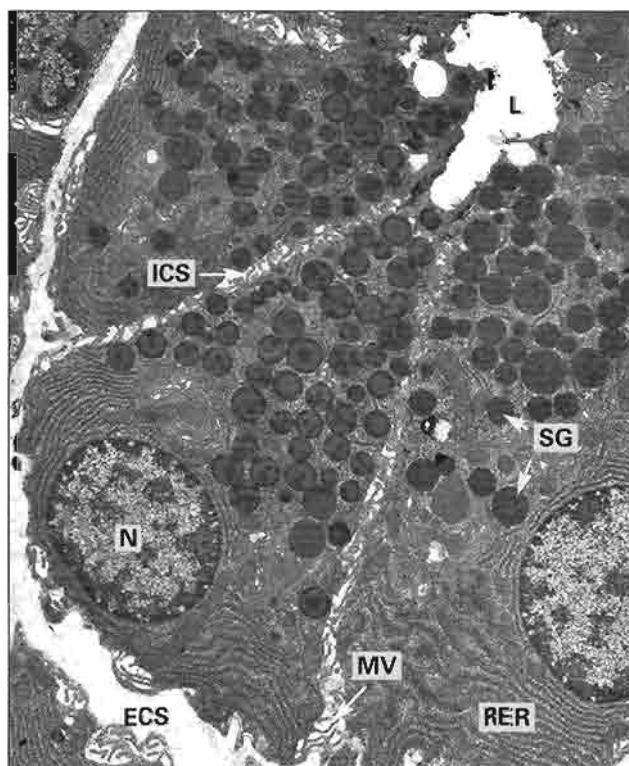


Fig 9-6 Electron micrograph of parotid gland acinar cells. (ECS) Extracellular space; (ICS) intracellular space; (L) lumen; (MV) microvilli; (N) nucleus; (RER) rough endoplasmic reticulum; (SG) secretory granules. (Original magnification $\times 4,400$.)

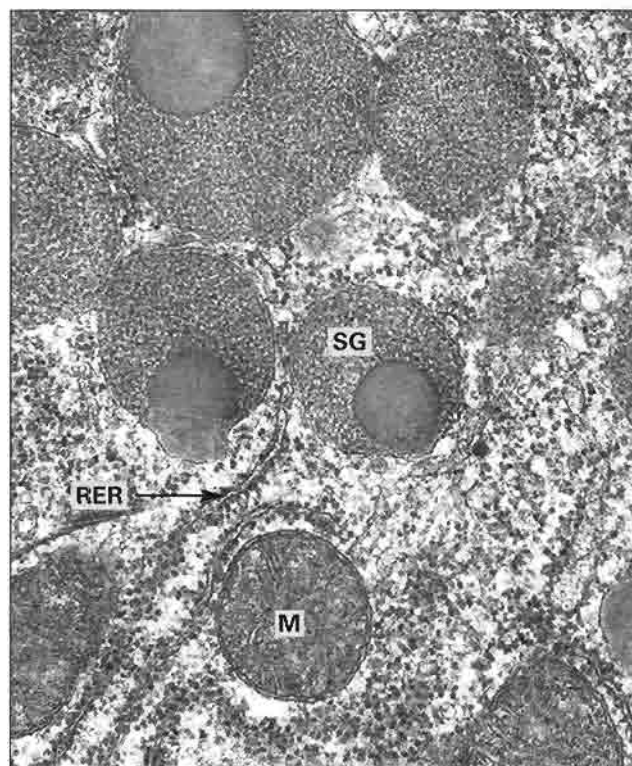


Fig 9-7 Enlarged view of the secretory granules in Fig 9-6 (M) Mitochondria; (RER) rough endoplasmic reticulum; (SG) secretory granules. (Original magnification $\times 22,000$.)

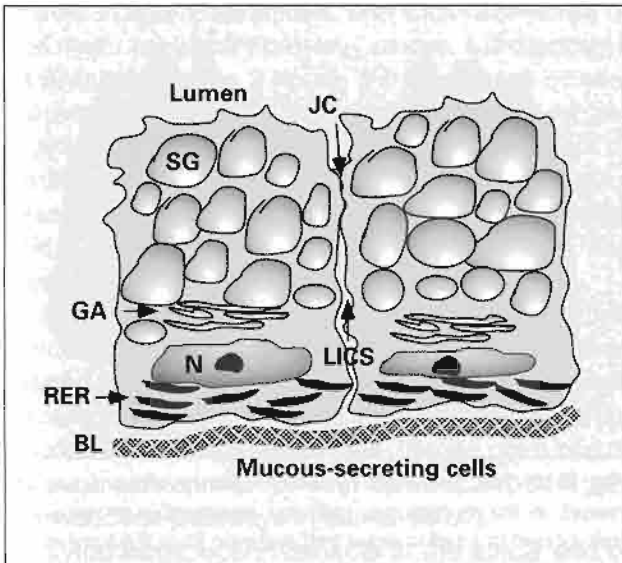


Fig 9-8 Structure of mucous cells. In the nonstimulated state, a mucous cell contains many large mucin-containing secretory granules (SG). Two thirds of the entire cell volume can be occupied by secretory granules. In this state, the Golgi apparatus (GA), the nucleus (N), and the rough endoplasmic reticulum (RER) appear compressed into the basal part of the cell. In general, the lateral intercellular space (LICS) is bordered by relatively straight cell membranes. A junctional complex (JC), consisting of a zonula adherens and a zonula occludens, is present at the proximal cell borders. (BL) Basal lamina.

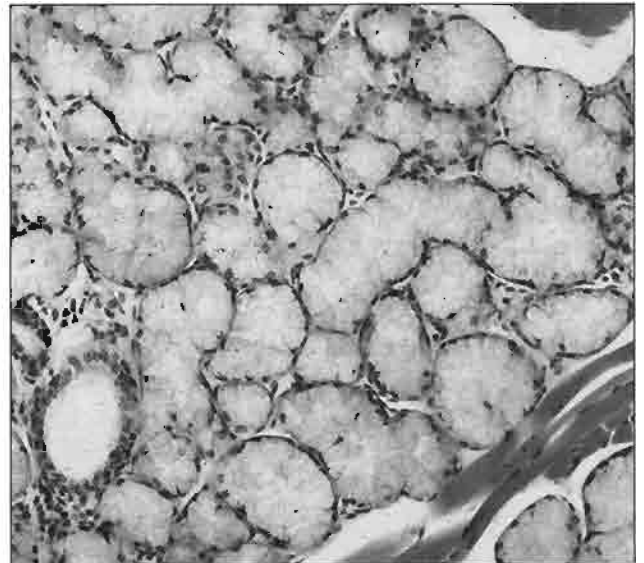


Fig 9-9 Histologic section of mucous secretory endpieces in a sublingual gland. Note the position of the flattened nuclei along the base of the secretory endpieces and the apparently "empty" or structureless cytoplasm. (Hematoxylin-eosin stain. Original magnification $\times 140$.)

ing the cell an overall pyramidal shape (see Fig 9-5). When grouped together, these pyramid-shaped serous cells form a round cluster, or acinus.

Serous cells are the last to differentiate and therefore they occupy a place distal to mucous cells of mixed secretory endpieces. This is best exemplified in the submandibular gland, where many secretory endpieces contain both mucous and serous cells. Here the serous cells are attached at the very end of the secretory endpiece in the form of a "demilune." Serous cells of a demilune discharge their secretions into intercellular canaliculi that communicate with the lumen through lateral intercellular spaces between the mucous cells.

The serous cells of salivary glands have much in common with the serous cells of the airway mucosal glands. These serous cells have been compared to immobilized neutrophils because they both secrete a wide variety of antimicrobial factors.

Mucous cells

The microanatomic appearance of the mucous cell varies with the stage of the secretory cycle. In a cell depleted of its secretory granules, the nucleus expands and occupies a more central location in the cell. The RER and Golgi complex expand in preparation for the synthesis and packaging of new salivary components. Cells in this stage can be mistaken for serous cells.

Mature, unstimulated mucous cells contain a full supply of secretion granules (Fig 9-8). In routine sections, unstimulated mucous cells are columnar in outline, and the apical two thirds of the cell appears empty (Figs 9-8 and 9-9). During routine tissue preparation, the granule membranes are ruptured, causing the mucins to undergo expansion and hydration. The small amount of protein that remains in the secretory granules is preserved as a delicate web of stainable material. The net result is an empty or poorly stained apical cytoplasm.

The nucleus of a resting mucous cell is usually flattened, densely stained, and pushed toward the base of the cell^{32,37} (see Figs 9-8 and 9-9). A small amount of RER located adjacent to the nucleus accounts for the basophilia of the basal cytoplasm. A characteristic feature of mucous cells is their distinct lateral borders. This is due to the relative absence of microvilli on the lateral cell surfaces. As a result of their columnar shape, mucous cells cluster in a tubular configuration, bordering a rather wide and well-defined central lumen.

Mucous acinar cells secrete mucins, the main component of the jellylike adherent layer that covers the surfaces of the oral cavity. Mucins account in large measure for the lubricating effect of saliva, essential to swallowing and speech. Two classes of mucins have been characterized: the large mucins (MG1) and the smaller mucins (MG2). The MG1 mucins are better at coating (lubricating) surfaces, while the smaller and more soluble MG2 mucins exert antibacterial and antiviral actions.

Tabak³⁸ has reviewed the structure and function of the salivary mucins. Both cholinergic and β -adrenergic neurotransmitters are capable of effecting mucin granular discharge.³⁹

Secretion of Saliva

Protein phase

Secretory proteins are transported from the RER to the Golgi apparatus in coated intermediate vesicles. In the Golgi complex, the intermediate vesicles fuse with cisternae of the forming face of the Golgi apparatus. Salivary secretory granules are formed from condensing vacuoles arising from mature Golgi cisternae.^{28,29,40}

During transport from the cis-Golgi network to the mature trans-Golgi network, the salivary secretory proteins undergo glycosylation and sulfation. From the trans-Golgi network, proteins follow one of two pathways to the external milieu, the constitutive (vesicular) pathway or the regulated (storage granule) pathway⁴¹ (Fig 9-10).

Although the vesicles of the constitutive pathway are mainly involved in transporting cell membrane proteins to the apical and basolateral plasma membrane, they appear to contain some secretory proteins. The constitutive pathway accounts for a relatively small but constant release of salivary proteins.⁴¹⁻⁴³ The constitutive pathway does not require activation by neurotransmitters nor is it blocked by parasympa-

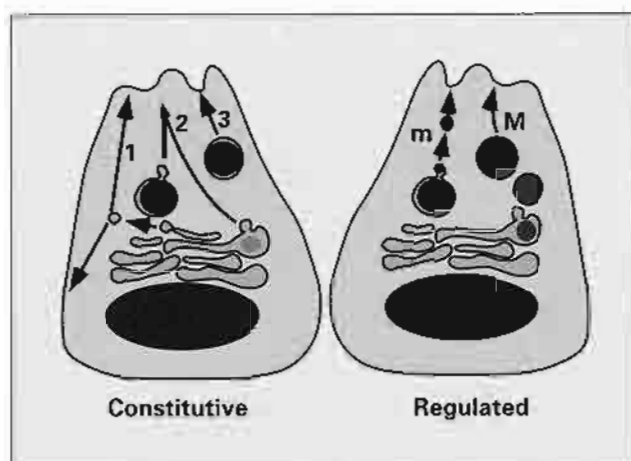


Fig 9-10 Two pathways by which salivary proteins are secreted. In the constitutive pathway, nongranule proteins are transported in small vesicles that originate from the trans-Golgi network (1). A small percentage of granule proteins are released from immature secretion granules (2) or by direct fusion of a secretion granule (3). Ninety percent of salivary proteins are stored in granules that form the neurotransmitter-regulated pathway. A minor (m) fraction of these proteins is released in small vesicles that originate from immature secretion granules, while the major (M) fraction is released by granule exocytosis. (Adapted from Castle and Castle⁴³ with permission.)

tholytic or sympatholytic agents. However, there is evidence that under low levels of calcium mobilization and parasympathetic nerve stimulation there is amplification of vesicular transport.⁴¹

In the classic constitutive pathway, proteins that are not destined to be stored leave the trans-Golgi network in small vesicles and are transported to the cell surface (see Fig 9-10). In a second constitutive-like pathway, some proteins are segregated in vesicles that bud from maturing secretory granules. Presumably these are proteins that fail to, or have yet to, be condensed into the granular cargo. Finally, a very small number of mature secretory granules may undergo unstimulated fusion, thereby contributing their cargo of proteins to the constitutive secretions (see Fig 9-10).

The regulated secretory pathway involves the storage of secretory proteins until the cell receives appropriate stimuli in the form of β -adrenoceptor agonists^{41,43,44} (see "Signal transduction pathways in acinar cells," later in this chapter). Stimulation of β -adrenoceptors activates the major regulatory pathway, involving a rapid release of granules by fusion to the luminal cell surface.⁴⁵ The luminal surface includes the membrane lining the lumen and

the intercellular canaliculi. Maximum activation of the major regulatory pathway causes full discharge of granules in 1 to 2 hours. Smaller doses of neurotransmitters appear to cause the release of low levels of secretory proteins by the formation of small vesicles that bud from maturing secretory granules in a mechanism similar to the second constitutive pathway (see Fig 9-10). This pathway has been called the *minor regulatory pathway*.⁴³

Secretion of mucin from mucous cells occurs following both cholinergic and β -adrenergic stimulation. Activation of cholinergic and α -adrenergic receptor induces some secretion of protein, particularly amylase by the parotid gland, presumably through activation of protein kinase C (see "Signal transduction pathways in acinar cells").

Cytoplasmic actin filaments at the apical end of the cytoplasm may act as a barrier to block the contact between granules and the cell membrane in the unstimulated cell. Thus, one of the first steps in the exocytosis of storage granules involves either a redistribution of the apical actin filaments or changes in the association of filaments to secretory granules.⁴⁵ Granular discharge begins about 10 to 15 seconds after stimulation by isoproterenol (β -adrenergic agonist).⁴⁵ It has been suggested that a fraction of the secretory granules are already docked to the luminal membrane.

Granule transport, docking, and fusion involve special proteins: soluble *N*-ethylmaleimide-sensitive fusion attachment proteins (SNAPs), SNAP receptor proteins, fusion proteins, and cytoplasmic guanosine triphosphate (GTP)-binding proteins (see chapter 2). These proteins have been best characterized in neuronal secretion but are found in acinar cells and are suspected to function in salivary secretion as well.⁴⁵⁻⁴⁸

Electron microscopic studies of granular discharge indicate that granules develop pseudopodia, which project toward the cell membrane and adjacent granules.⁴⁹ Formation of pseudopodia by secretory granules can be triggered in vitro by the conversion of adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP) and the activation of protein kinases, two mechanisms operating in vivo in the signal transduction pathways of the intact gland (see "Signal transduction pathways in acinar cells").⁵⁰ Contact between the granule pseudopod and the plasma membrane leads to the formation of a fusion pore and the discharge of the granular cargo into the lumen. These final steps involve phosphorylation of granule membrane proteins by protein kinase A and regulation by calmod-

ulin and the local intracellular concentration of Ca^{++} .⁵⁰⁻⁵²

Using a cell-free system, Mizuno-Kamiya et al^{53,54} have demonstrated that isolated plasma membranes from parotid glands can evoke the release of amylase from secretory granules without any additional factors. This process requires a newly identified form of phospholipase A2, present in the secretory granule membrane. Adenosine triphosphate activates this granule-associated phospholipase A2 in a Ca^{++} -dependent mechanism. Although these results suggest that exocytosis may be regulated by components already in place on the luminal and granule membranes, further in vivo studies are needed to confirm this mechanism.

The formation of secretory granules involves a maturation process requiring the condensation of secretory proteins.⁴³ Mature granules contain proteins that are approximately 20 times more concentrated than when they left the trans-Golgi network. The condensation of proteins is a complex and poorly understood process. Proposed mechanisms for concentrating secretory proteins include shielding the charges of secretory proteins by secondary sulfated molecules such as glycosaminoglycans, and/or the formation of calcium bridges between negatively charged residues.⁴³ The large, and highly charged, mucin polymers must undergo condensation and stabilization by interaction with calcium ions and positively charged organic molecules⁵⁵ (Fig 9-11).

The heterogeneous content of most salivary secretory granules becomes evident during the condensation process. Sequestration of various proteins inside the granules by homeotypic condensation creates various patterns of protein distribution. These patterns are evident in electron micrographs.²⁸ Comparative studies of salivary secretory granules of numerous species of animal indicate that protein aggregation patterns are, to a certain degree, species specific.

During the exocytosis of secretory granules from mucous cells, the highly charged mucins undergo rapid expansion as the calcium content of the granule is diluted by contact with fluid of the lumen. Hydration of mucin polymers is explained in part by a local Donnan's equilibrium effect, as water and small positively charged molecules are drawn into the inner domain of the expanding polymer⁵⁵ (see Fig 9-11). Some proteins are discharged in a semi-crystalline form. Under normal circumstances, the crystals are rapidly dissolved and the proteins are dispersed in the salivary fluid. However, in patients

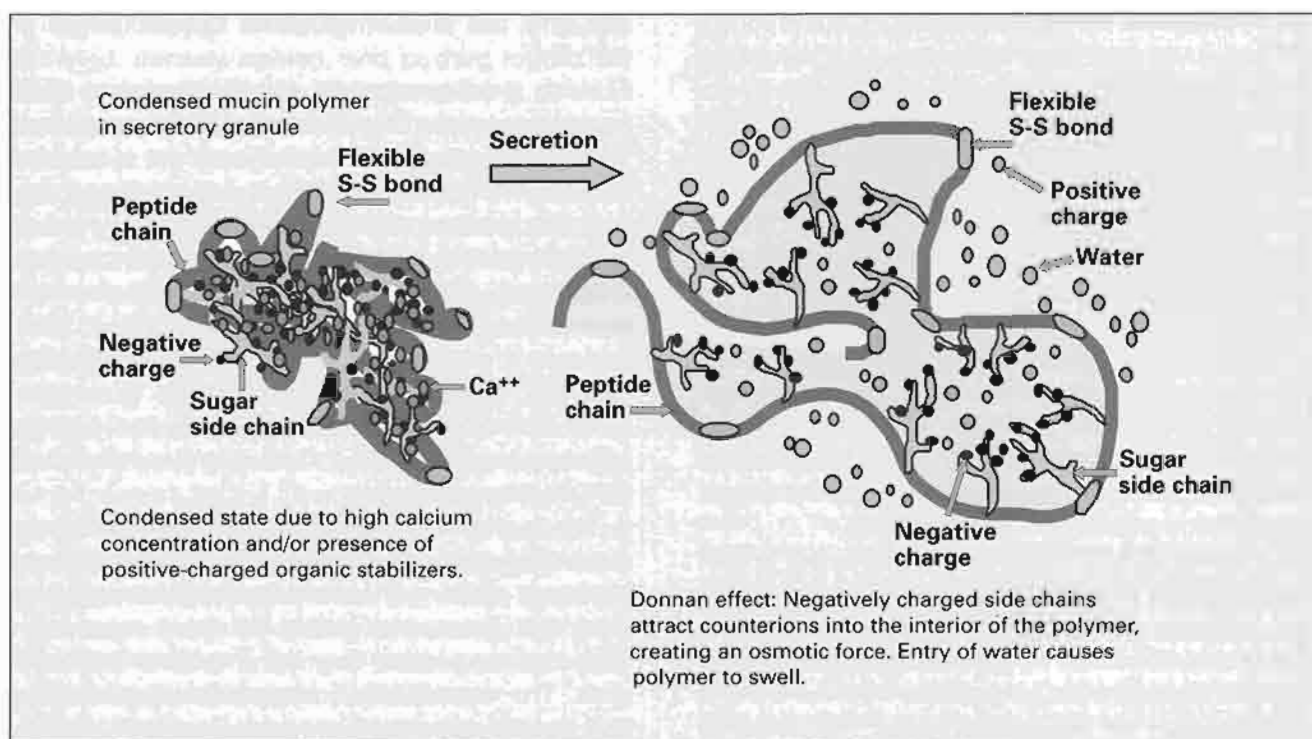


Fig 9-11 Expansion of a mucin polymer from its condensed form in the secretory granule to its hydrated state in the extracellular fluids.

who have cystic fibrosis, in whom the calcium concentration in the saliva is elevated, the granules do not dissolve as quickly, resulting in the formation of thick and viscous saliva.

After expulsion of granules, the excess surface membrane is retrieved by endocytosis. Formation of endosomes and the subsequent processing of plasma membrane components occur via the Golgi-lysosomal network.

Fluid phase

Water flows through and between the acinar cells in response to the osmotic gradient created by the transport of NaCl across the epithelium. Both Na^+ and Cl^- enter acinar cells through a $\text{Na}^+\text{-K}^+\text{-Cl}^-$ cotransporter located in the basolateral membrane^{33,56,57} (see "Signal transduction pathways in acinar cells"). This cotransporter is activated by the release of Ca^{++} from intracellular stores, following the activation of phospholipase C and the generation of the second messenger inositol triphosphate, and by an increase of cAMP generated by vasoactive intestinal polypeptide stimulation.^{58,59}

The electrochemical gradient for Na^+ drives the cotransporter activity, allowing Cl^- to be transported above its electrochemical gradient.³³ Potassium ions also exit through a Ca^{++} -activated potassium channel in the basolateral membrane.^{33,60} Chloride ion is transported across the apical (luminal) membrane through a Ca^{++} -activated chloride channel and the cystic fibrosis conductance regulator.^{61,62} The cystic fibrosis conductance regulator acts not only as a chloride channel but also as a regulator of ATP and Na^+ transport. Sodium ions are pumped out of the acinar cell into the paracellular compartment by the $\text{Na}^+\text{-K}^+$ -adenosine triphosphatase (ATPase), or Na^+ pump.³³ Entry of Cl^- into the lumen generates a transepithelial potential that pulls sodium ions across the epithelium through the paracellular route.^{33,56}

Water moves across the acinar cell through specific water channels called *aquaporins*.⁶³ Aquaporins have been identified in the membranes of many glandular epithelial cells, including the apical membranes of salivary acinar cells.^{64,65} Increased intracellular calcium stimulates the translocation of aquaporin 5 from the cytoplasm to the luminal plasma membrane.⁶⁶ In transgenic mice lacking aquaporins,

the production of saliva is reduced by 60% following stimulation with pilocarpine (cholinergic agonist).⁶⁷

Water also flows in the paracellular pathway through the leaky zonula occludens junctions.^{68,69} Tracer experiments with microperoxidase indicate that the permeability of the acinar cell zonula occludens may be regulated by cholinergic stimulation.⁷⁰

Resynthesis of Proteins

Secretion of salivary proteins is followed by new protein synthesis to replenish the supply of secretory granules.⁴¹ Resynthesis, like the secretory process, is mainly regulated by neurotransmitter stimulation of signaling pathways. The β -adrenoceptor agonist isoproterenol increases amino acid uptake and protein synthesis in acinar cells.^{71,72} Isoproterenol also stimulates DNA synthesis and proliferation of acinar cells.⁷³ In vitro studies have demonstrated that β -adrenoceptor agonists increase protein synthesis via a cAMP second messenger pathway.⁴¹ Low levels of cholinomimetics and calcium-mobilizing agents also promote protein synthesis in salivary acinar cells. However, at high levels these substances inhibit secretion.⁴¹

An increase in reflex neural stimuli to the glands during mastication triggers a phase of new protein synthesis. The importance of reflex neural stimulation is illustrated in experimental animals by the atrophy of glandular tissue that follows long-term consumption of liquid diets.

Increased protein synthesis may result from increased gene transcription, from increased translational activity of existing messenger ribonucleic acid (mRNA), or from the stabilization of mRNA and ribosomes. The β -adrenoceptor agonist-cAMP pathway increases transcription of salivary secretory protein genes. Not all salivary secretory proteins are similarly regulated; for example, amylase appears to be regulated at the translational level, while proline-rich proteins are mainly regulated at the transcriptional level.

Composition of Saliva

Salivary proteins

Saliva contains a mixture of proteins that have evolved to initiate the digestion of food and to protect the oral tissues from viral and microbial infection (see Table 9-1). The major digestive action of saliva is a result of its amylase content. Cystatins (inhibitors of cysteine pro-

teases) inhibit bacterial and neutrophil proteases.⁷⁴ Proline-rich proteins and cystatins inhibit virus replication by interfering with their ability to enter host cells.⁷⁵ Secretory IgA helps to prevent bacterial cell adhesion to tooth surfaces and epithelial cells.^{76,77} Lysozyme, lactoferrin, and peroxidase limit bacterial growth by disrupting cell walls and interfering with metabolism.^{78,79} Statherins and proline-rich proteins promote enamel remineralization while minimizing the precipitation of calcium phosphate salts in salivary ducts.⁷⁴

Growth factors

Vascular endothelial growth factor has been localized in human parotid and submandibular acinar cells.^{80,81} Increased secretion of growth factors in saliva following oral surgery suggests that this response may promote wound healing in the mouth.³ Growth factors and some salivary proteins can gain entry into the bloodstream by an unknown mechanism. For example, nerve growth factor is secreted into the bloodstream by salivary glands in mice during periods of increased aggressive behavior.⁸²

Saliva also contains numerous growth factors and other peptides that can modulate the inflammatory-immune response.⁸³ Nerve growth factor potentiates proliferation of T and B lymphocytes. Epidermal growth factor and transforming growth factor α increase fibroblast proliferation and angiogenesis. Proinflammatory cytokines, interleukin 1 β (IL-1 β), and IL-6 are also stored in salivary secretion granules.⁸⁴

Mathison et al⁸³ have suggested that, because of its secretion of mediators, the submandibular gland be considered a component in the neuroendocrine regulation of the immune response. In this view, the autonomic modulation of salivary production of regulatory peptides represents one pathway for regulating inflammatory and wound-healing processes.

Nonsecretory Components of the Salivary Glands

Myoepithelial cells

As their name implies, myoepithelial cells are of epithelial stem cell origin and are specialized for contraction.^{6,85,86} Myoepithelial cells are located in the space between the basal lamina and the epithelial cells of the secretory endpieces and the proximal segments of the ducts (including striated and granular ducts).^{6,87-89} In some animals they are also present on the proximal part of the excretory ducts.

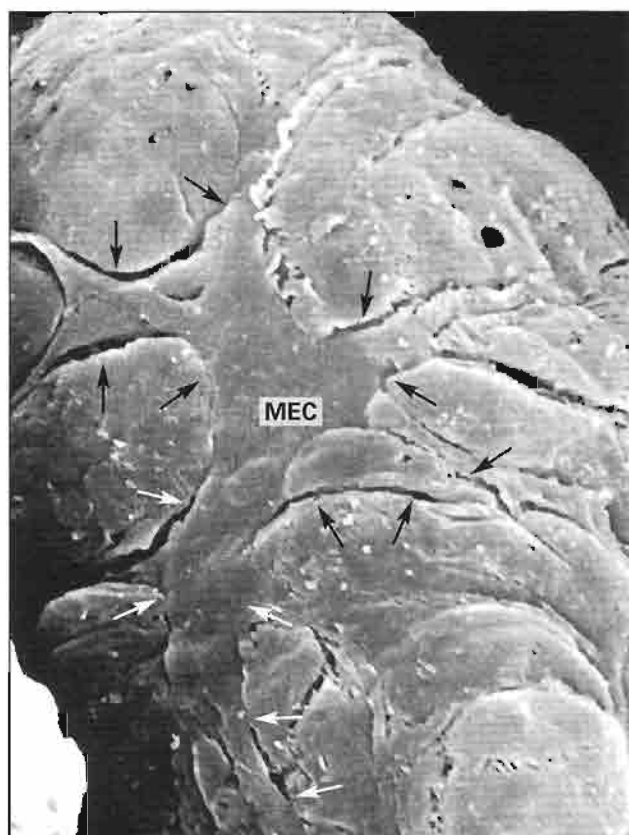


Fig 9-12 Scanning electron micrograph of a stellate myoepithelial cell (MEC) on the surface of an acinus. Tissue was prepared with collagenase and hydrochloric acid for the removal of connective tissue and basement membrane. Arrows outline the borders of a myoepithelial cell. (Reprinted from Riva et al¹¹ with permission from John Wiley & Sons.)

Myoepithelial cells associated with acinar cells have a polygonal cell body with numerous branching cytoplasmic processes that are closely applied to the external surface of the epithelial cells^{85,89,90} (Fig 9-12). Myoepithelial cells associated with ductal elements appear spindle shaped; their long axis is oriented parallel to the long axis of the duct. Firm contact is made between epithelial cells and myoepithelial cells by the formation of numerous desmosomes.⁸⁷ Gap junctions connect contiguous myoepithelial cells.³⁴ In some species, gap junctions between myoepithelial cells and mucous acinar cells have been described.

Myoepithelial cells contain numerous actin and myosin filaments arranged in bundles parallel to the long axis of their cell processes. The cell membrane facing the basal lamina contains numerous endocytotic pits, or caveolae, and stains intensely for alkaline phosphatase, ATPase, and adenylyl cyclase ac-

tivity.^{91,92} In contrast, Cutler et al⁹³ were unable to demonstrate alkaline phosphatase staining of myoepithelial cells in human parotid and submandibular glands.

Myoepithelial cells are difficult to identify in routine hematoxylin-eosin-stained sections. They are best studied with histochemical stains, such as those that demonstrate alkaline phosphatase and ATPase activity, or with immunocytochemical stains for actin and/or myosin.^{6,85,90,94,95} The distinct internal morphology of the myoepithelial cell is evident in transmission electron micrographs, while their three-dimensional structure is best appreciated in scanning electron micrographs (see Fig 9-12).

Myoepithelial cells are innervated by parasympathetic and sympathetic nerve fibers.⁸⁷ Nerve stimulation causes the myoepithelial cells to contract and thereby exert tension on acinar and ductal cells. Contraction of myoepithelial cells is inhibited by α -adrenoceptor, but not β -adrenoceptor, blockade. Although this contraction can help to expel secretion granules from the secretory cells, its other function is to resist secretory pressure and to support and stabilize the secretory cells against increased luminal pressures that develop during high rates of secretion.⁸⁷

Intercalated ducts

Intercalated ducts lead directly from the secretory endpieces. Several secretory endpieces may join a single branched intercalated duct. All intercalated ducts are intralobular.

The cells of the intercalated duct are low cuboidal in outline and are characterized by a centrally placed nucleus and clearly visible cell boundaries.

The cells of the intercalated ducts contain relatively few cytoplasmic organelles, indicative of low levels of synthetic and secretory activity.⁸⁹ At the electron microscopic level, the cells are observed to contain a small amount of RER and a poorly developed Golgi apparatus. The small number of secretory granules that are present suggests that a small amount of salivary protein is added to the saliva in the intercalated duct.⁴⁹

The great variation in salivary gland structure exhibited in mammals extends to the intercalated ducts. Differences in thickness, branching, and granule protein contents have been reviewed recently.⁹⁶

One suggested function of this part of the salivary duct is to provide a reservoir of progenitor cells capable of regenerating the more specialized components of the gland.⁹⁷⁻⁹⁹ In sections of normal healthy

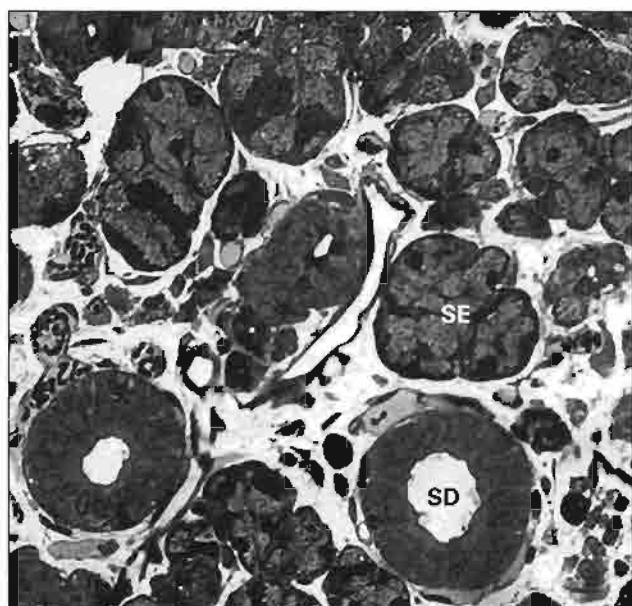


Fig 9-13 One-micron section of a rat submandibular gland illustrating two striated ducts (SD), cut in cross section, and several secretory endpieces (SE). (Toluidine blue stain. Original magnification $\times 240$.)

tissue prepared for routine histologic examination, it may be difficult to identify the intercalated duct cells. However, in inflamed tissue, where the secretory cells have undergone degeneration, the intercalated duct cells usually stand out. The highly differentiated segments of the gland appear more susceptible to toxic conditions and are the first to undergo necrosis, while the more resistant cells of the intercalated ducts persist. Although the intercalated duct may represent a primary reservoir of relatively undifferentiated cells, recent studies indicate that acinar cells are able to divide and may participate in regeneration of secretory endpieces, including intercalated ducts.¹⁰⁰

Striated ducts

Striated ducts have an intralobular distribution.⁶ Striated ducts are lined by columnar cells arranged in a simple and/or pseudostratified configuration (Figs 9-13 and 9-14). A large, centrally positioned nucleus and cytoplasmic basal striations make these cells easily identifiable in histologic sections. The basal cell surface is highly infolded, creating vertical sulci

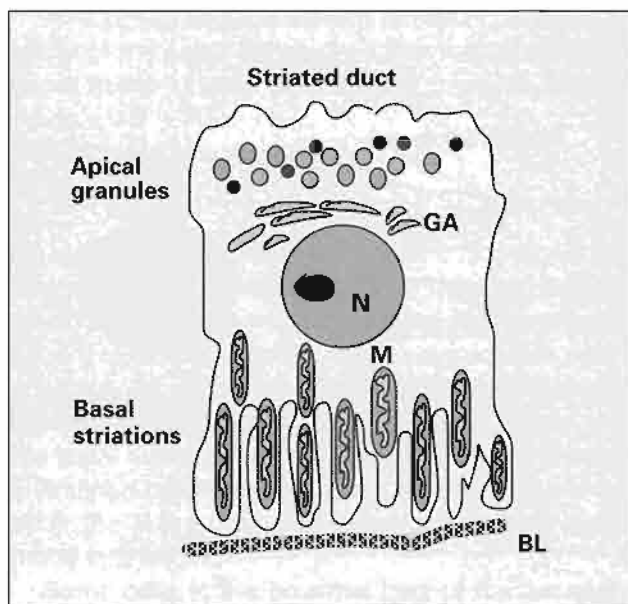


Fig 9-14 Epithelial cell of a striated duct. Basal striations formed by cytoplasmic infoldings and longitudinally oriented mitochondria (M) dominate the basal part of the cell. The nucleus (N) is typically large, round, and centrally located. Numerous small granules are concentrated beneath the apical surface. The Golgi apparatus (GA) is relatively inconspicuous. (BL) Basal lamina.

and a large surface area juxtaposed to the underlying stromal connective tissue.^{6,11,68,101} Numerous mitochondria assume an elongated shape and an alignment perpendicular to the base of the cell, parallel to the infolded segments of the cell membrane (see Fig 9-14). Alignment of mitochondria in the cytoplasmic compartments formed by the basal infoldings accounts for the cell's characteristic radial eosinophilic striations. There is extensive interdigitation or interfoliation of the basal and lateral infoldings between adjacent cells.^{11,68,101}

As saliva flows through the striated duct, it becomes hypotonic as Na^+ and Cl^- are reabsorbed in excess of water.^{6,56,69} The epithelial lining of the ducts is highly impermeable to water. Na^+ enters duct cells across the luminal membrane via Na^+ channels and a Na^+/K^+ exchanger.⁶⁹ The basal membrane contains Na^+/K^+ -ATPase activity, responsible for the active transport of sodium ions across the plasma membrane into the extracellular space of the connective tissue⁶⁹ (Fig 9-15). The net effect is reabsorption of NaCl without water, thereby rendering the saliva slightly hypotonic.

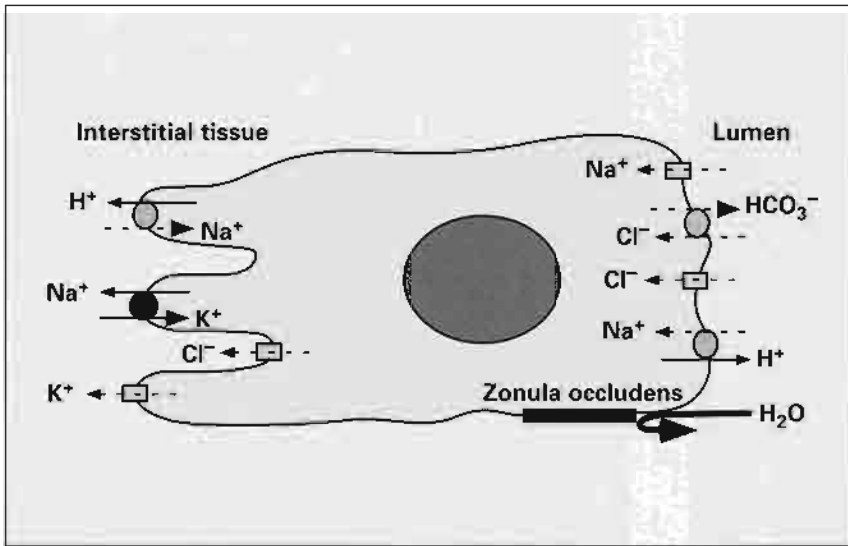


Fig 9-15 Electrolyte transport across cells of the striated duct. Na^+ enters across the luminal membrane via Na^+ channels and Na^+-H^+ exchanger. Chloride enters across the luminal membrane through Cl^- channels and through a less well-documented transporter (the Cl^- - HCO_3^- exchanger). Na^+ is actively extruded at the basolateral membrane via Na^+-K^+ -adenosine triphosphatase. Cl^- and K^+ channels permit passive diffusion of their respective ions into the interstitial space. A Na^+-H^+ exchanger is also present in the basolateral membrane. The net effect is the adenosine triphosphatase-driven reabsorption of Na^+Cl^- without water. (Adapted in part from Poulsen.⁶⁹)

Bicarbonate is added to the saliva in the striated duct.^{56,102} Carbon dioxide diffusing into the cell is converted to HCO_3^- and H^+ via the activity of carbonic anhydrase. HCO_3^- is secreted into the saliva in exchange for Cl^- .¹⁰²

Immunocytochemical studies have shown that an anion exchanger located in the basolateral infoldings may be responsible for transporting H^+ out of the cell in exchange for Na^+ . Duct cells contain α - and β -adrenergic and cholinergic receptors. Parasympathetic and sympathetic nerve stimulation lead to alterations in electrolyte transport across the ducts.⁵⁶ A vacuolar-type H^+ -ATPase (H^+ pump) has also been localized in salivary duct cells.¹⁰³ In acidosis, the H^+ -ATPase shifts to the apical cytoplasm, suggesting a potential role for salivary glands in excreting H^+ into saliva.

In some species, including humans, the apical cytoplasm contains numerous small granules.⁶ Epidermal growth factor, fibronectin, secretory IgA, lysozyme, and kallikrein have been localized in these apical granules. Striated ducts have been identified by immunofluorescent microscopy as potential sites for the secretion of epidermal growth factor, fibronectin, lysozyme, kallikrein, and secretory IgA.

Micropuncture studies show that the initial saliva in the lumen of the secretory endpieces is isotonic and that it becomes hypotonic in the excretory ducts. The initial isotonic secretory fluid contains high sodium and low potassium concentrations. Reabsorption of sodium in excess of water within the stri-

ated ducts, and to some degree in the initial segments of the excretory ducts, leads to hypotonicity of the final secretion (see Fig 9-15).

The movement of water across the duct lining is restricted because the epithelial cells are highly impermeable to water and the intercellular spaces are sealed by zonula occludens junctions. When parasympathetic nerve stimulation is decreased, the flow of saliva is decreased. Under these conditions the saliva remains in contact with the cells of the striated and excretory ducts for a longer period of time, more sodium and chloride ions are reabsorbed, and the saliva becomes more hypotonic. In contrast, high flow rates lead to more isotonic saliva. In some animals, sodium restriction can lead to compensatory hypertrophy of the striated ducts to maximize sodium retention.

Granular ducts (granular convoluted ducts)

Granular ducts are not present in human salivary glands. They represent a modified striated duct located between the intercalated segment and typical striated duct cells. They are formed by columnar cells filled with large secretory granules that stain intensely with hematoxylin and basic dyes (Fig 9-16).

The granules have been shown to contain nonspecific proteases, such as kallikrein and renin.^{6,42,104-106} Nerve growth factor, epidermal growth factor, trans-

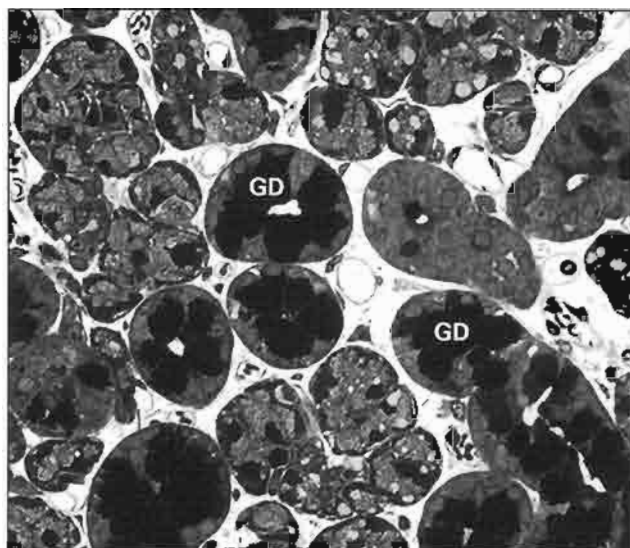


Fig 9-16 One-micron section of a rat submandibular gland containing several granular ducts (GD) filled with dense granules, amid several secretory endpieces. (Toluidine blue stain. Original magnification $\times 240$.)

forming growth factor α , hepatocyte growth factor, insulin-like growth factor, and mesodermal growth factors are also found in the granular duct cells.^{6,107-109} Among the earliest indications that salivary glands produced growth factors was the finding that a protein (later identified as nerve growth factor) isolated from the mouse submandibular gland induces accelerated epidermal proliferation and keratinization.¹¹⁰ Through its ability to increase epidermal keratinization, it promoted early tooth eruption and eyelid opening.¹¹¹

Granular ducts are highly developed in rats and mice, especially in mature males.¹¹² Castration and hypophysectomy cause a decrease in the number of granular ducts and a concomitant decrease in the level of epidermal growth factor and nerve growth factor in blood and saliva. Androgens, thyroxine, and adrenal cortical hormones stimulate development of granular ducts. Secretion of nerve growth factor and kallikrein increases after adrenergic stimulation.

Although a causal link between the presence of growth factors in saliva and the rapid wound-healing response of oral mucosal surfaces has not been firmly established, it is reasonable to suspect that such a relationship exists. It is well known that epidermal growth factor and hepatocyte growth factor stimulate proliferation of keratinocytes. Thus, when animals lick their wounds, they not only perform mechanical debridement but also deliver salivary proteolytic enzymes, growth factors, and antibacterial substances to the wound bed.

Although granular ducts are not found in humans, some of the same substances that they contain are found in the striated duct cells of primates and humans.

Excretory ducts

As saliva leaves the striated ducts, it is drained into larger interlobular excretory ducts. The proximal segments of the excretory ducts are lined by simple and pseudostratified epithelia. Some cells contain apical granules. Basal cells are tucked between the taller columnar cells.¹¹ Both cell types are in contact with the basal lamina. The main excretory duct is lined by a stratified columnar epithelium. At the orifice of the duct, the lining gradually becomes stratified squamous epithelium.

Some cells in the proximal part of the excretory duct have basal eosinophilic striations and may participate to a limited extent in the reabsorption of sodium. Mucous goblet cells, scattered among the lining cells, release mucins along the luminal surface of the distal segments of the main excretory duct.

Oncocytes

These cells are strongly eosinophilic because of their very high concentration of mitochondria.^{6,96} They are present in small numbers in secretory endpieces and ducts. Oncocytes increase in number with age and sometimes proliferate to give rise to tumors (oncocyctomas). Their function is unknown.

Duct-associated lymphoid tissue

Morphometric analysis of the tissue composition of the oral mucosa has shown that up to 1% to 5% of its volume is composed of lymphoid tissue.¹¹³ The bulk of this lymphoid tissue is found in close relationship to the ducts of the minor salivary glands of the soft palate, vestibular surfaces of the lips, floor of the mouth, and the ventral surface of the tongue.¹¹⁴ Clusters of lymphocytes and plasma cells surround the deepest segments of the excretory ducts. Typical germinal centers are formed in these lymphoid aggregations.

At birth, the newly formed minor salivary glands are devoid of lymphoid tissue. In the infant, as the glands become exposed to foreign substances, there is a gradual increase in the presence of duct-associated lymphoid tissue.¹¹⁵ Peak development of duct-associated lymphoid tissue occurs in young adulthood; thereafter, development declines with age.

Antigenic stimulation of the glandular stroma occurs via retrograde movement of foreign molecules within the ducts during periods of reduced salivary stimulation. Experiments in animals have shown that tracer molecules placed at the surface of the oral mucosa, next to the orifice of the main duct, gain access and penetrate deep into the ducts of the minor glands.¹¹⁶ Additional studies in animals have demonstrated that immunization with purified bacterial proteins via the intraductal route of the parotid gland produced increased levels of specific secretory IgA in saliva. The parotid and submandibular glands are also an important source of secretory IgA.¹¹⁷ The duct-associated lymphoid tissue system of the minor glands is believed to be the major source of salivary secretory IgA in humans, responsible for antibacterial and antiviral protection.

Structure of the Major Glands

The parotid gland contains mostly serous acinar secretory endpieces.⁴⁹ The junction between the secretory endpieces and the intercalated ducts is sharply defined. The intercalated ducts are relatively long and branched.⁶ The striated ducts are conspicuous. In humans, the main excretory duct, first described in 1661, is named after its discoverer, Niels Stensen. It joins the oral cavity adjacent to the maxillary first molars.

In general, the secretion of the parotid gland is watery and rich in protein. The acinar cells have a significant number of infoldings on their basal and lateral surfaces, a condition correlated to the production of the primary fluid component of the secretion. Up to 70% of the parotid salivary protein is a proline-rich protein believed to have an important role in preventing enamel dissolution. Peroxidase and amylase are also found in high amounts in parotid secretions.

The submandibular gland is a mixed gland with serous acini predominating over the mucous elements. Numerous mucous endpieces are capped by serous demilunes. Long and well-defined striated ducts are conspicuously present. The main duct is Wharton's duct, first described by Thomas Wharton in 1659. It empties into the mouth at the base of the tongue near the mandibular incisors. The secretion of this gland contains more mucous than that of parotid gland saliva; thus it is slightly more viscous.

The sublingual gland is a mixed gland with abundant mucous-secreting endpieces. Some are capped by serous demilunes.^{118,119} The secretory endpieces empty into short intercalated ducts. The striated

ducts have fewer basal striations and are not highly developed.¹¹⁹

The anterior lingual (Blandin-Nuhn) glands are located on the ventral surface near the lingual frenum. They are made up almost entirely of mucous-secreting endpieces with seromucous demilunes.¹²⁰ These glands have no connective tissue capsules. Distinct striated ducts are not present; however, individual cells with basal striations are dispersed in the excretory ducts.

The glands of von Ebner, or posterior lingual glands, are composed of serous acini.²⁹ They are the source of a salivary fluid rich in lipase that flows into the groove surrounding the circumvallate papillae. A specific protein, von Ebner's gland protein, has been identified as a member of the lipocalin family of lipophilic-ligand carrier proteins.¹²¹ It may play a co-factor role in chemical sensing as a transporter of lipophilic tastants, either to concentrate them at the receptor site or to clear them from the taste pit. Von Ebner's lipocalin protein inhibits cysteine proteinases.¹²² This cystatin-like activity may have an antimicrobial effect in the circumvallate groove.

A third group of lingual glands (Weber's), located in the pharyngeal base of the tongue, are mucus-secreting glands.

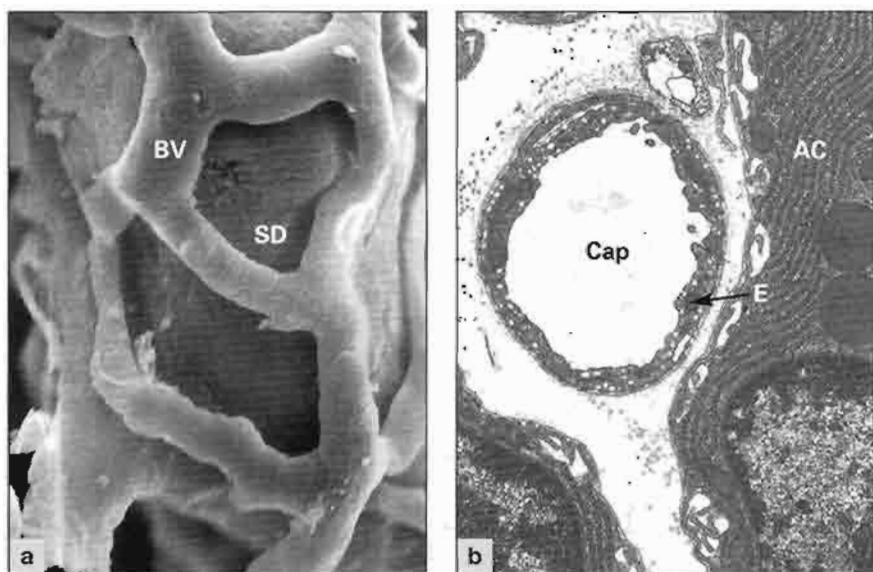
The minor salivary glands are usually named after their location in the oral mucosa, such as the labial, lingual, palatal, and buccal glands. With the exception of the larger glands located in the body of the tongue, most mucosal glands are about 1 to 3 mm in diameter and are located in the submucosal connective tissue beneath the lamina propria. Minor salivary glands are mainly mucous-secreting glands and a major source of secretory IgA.^{32,123}

A connective capsule surrounds the parotid, submandibular, and sublingual glands, a condition not found in the minor glands. Inflammation of the major glands produces painful swelling because nerve endings are compressed by the expansion of the parenchymal tissues against the capsule. The most frequent cause of salivary inflammation is bacterial or viral infection.

The secretory endpieces and ductal portions of the salivary glands receive a rich vascular supply. Fenestrated capillaries are in close apposition to the acinar cells and the cells of the striated ducts (Figs 9-17a and 9-17b). Autonomic control of vasodilation is coordinated to provide optimal fluid and ionic exchange in the stromal extracellular spaces adjacent to the secretory cells and ductal elements, especially those of the striated ducts.

Fig 9-17a Scanning electron micrograph of the blood vessel network (BV) juxtaposed on the outer surface of a segment of striated duct (SD). The stromal connective tissue has been removed by prior treatment with collagenase and hydrochloric acid. (Adapted from Riva et al¹¹ with permission from John Wiley & Sons.)

Fig 9-17b Electron micrograph of a capillary (Cap) in cross section adjacent to acinar cells (AC). (E) Endothelium. (Original magnification $\times 6,000$.)



Innervation and Neural Control of Salivary Secretion

Reflex stimulation of salivary glands plays a significant role in salivation. In humans, the smell and sight of food has only a minor effect on salivary flow. However, gustatory stimuli such as acidic and sour foods (lemon drops) trigger high rates of flow. The act of chewing stimulates salivation via reflex pathways activated by input from periodontal ligament mechanoreceptors. The brain stem neurons of the salivatory centers also receive positive and negative modulation from higher centers. For example, anxiety elevates sympathetic activity, reduces parasympathetic activity, and thereby reduces salivary flow.

Neuroanatomic pathways and the relative distribution of sympathetic and parasympathetic fibers to each of the major glands vary among species.¹²⁴⁻¹²⁶ There is also much diversity in the ultrastructural localization of nerve terminals vis-à-vis parenchymal cells and in the signal transduction events that occur in acinar cells. The following description is an attempt to present a model of glandular nerve function that fits most of the reported observations, as a basis on which to build knowledge through further study.

Electron microscopic studies have revealed epilemmal nerve endings in the interstitial connective tissue next to the basal lamina around secretory endpieces and ducts. There are also numerous intraep-

ithelial or hypolemmal nerve endings in the lateral intercellular spaces between epithelial cells¹²⁵⁻¹²⁷ (Figs 9-18a and 9-18b). The axons supplying the hypolemmal nerve endings lose their Schwann cell covering prior to traversing the basal lamina. Neurotransmitters are contained in small, 20-nm clear vesicles and dense-cored vesicles. The small, clear vesicles contain acetylcholine; the small, dense-cored vesicles contain norepinephrine; and the larger dense-cored granules, 40 to 80 nm in diameter, contain peptidergic neurotransmitters such as vasoactive intestinal peptide, calcitonin-gene-related peptide and substance P. Parasympathetic nerve endings contain cholinergic and peptidergic neurotransmitters, while sympathetic nerve endings contain noradrenergic neurotransmitters.

Salivary glands are innervated by parasympathetic and sympathetic nerves^{125,126} (Figs 9-19 and 20). Parasympathetic stimulation of the parotid gland arises from neurons located in the inferior salivatory nucleus of the brain stem. Peripheral axons travel in the root of the ninth cranial nerve, through the jugular ganglion, following the tympanic nerve and small superficial petrosal nerve, to the otic ganglion where they synapse with second-order neurons (see Fig 9-19). Postganglionic fibers leaving the otic ganglion proceed to the parotid gland along the auriculotemporal nerve. Parasympathetic stimulation from neurons in the superior salivatory nucleus travels along the chorda tympani branch of the facial nerve to the

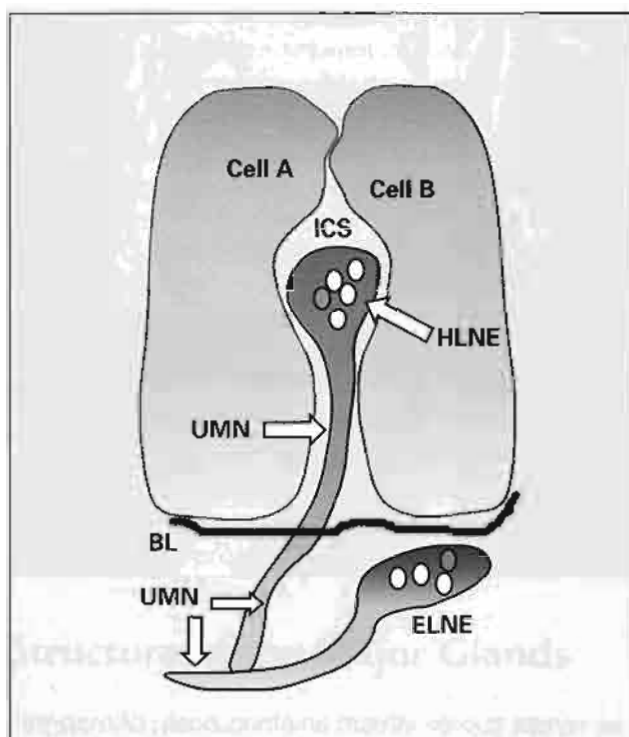


Fig 9-18a Relationship of nerve endings to cells in secretory endpieces and ducts. Unmyelinated nerves (UMN) pass through the basal lamina (BL) and terminate within the intercellular space (ICS) as bulblike hypolemmal nerve endings (HLNE) in juxtaposition to the cell membrane. Additional epitemmal nerve endings (ELNE) terminate in the connective tissue just beneath the basal lamina.

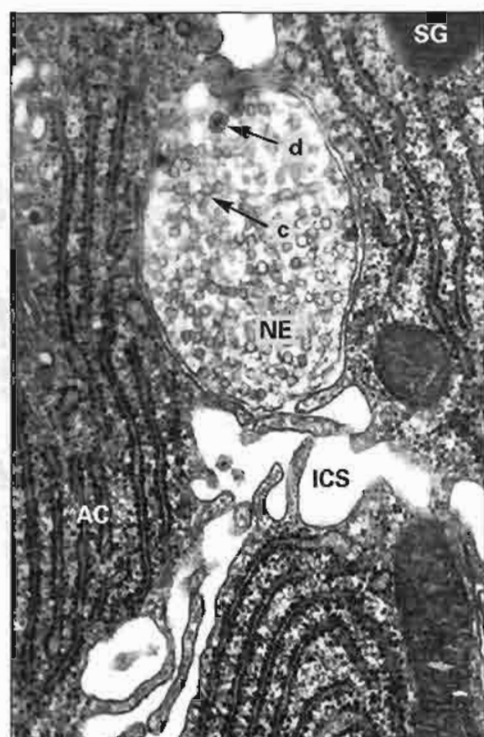


Fig 9-18b Electron micrograph of a hypolemmal nerve ending (NE) located between two acinar cells (AC). Note the abundance of clear (c) and dense (d) neurotransmitter vesicles. (ICS) Intracellular space; (SG) secretory granule. (Original magnification $\times 19,000$.)

submandibular (submaxillary) ganglion and then by postganglionic fibers that innervate the submandibular and sublingual glands.

Preganglionic sympathetic fibers leave the intermediolateral cell column in the first and second thoracic cord segments via the ventral root fibers to the superior cervical ganglion (see Fig 9-20). Postganglionic sympathetic fibers to all three glands travel from the superior cervical ganglion along the vascular supply to the otic ganglion and submaxillary ganglion. The sympathetic fibers pass through the ganglia without synapsing and course with parasympathetic branches to the individual glands.

When sympathetic nerves to a salivary gland are stimulated, both α - and β -adrenergic receptors are activated by the neurotransmitter, norepinephrine. Because there are more β -receptors than α -receptors on salivary secretory cells, the net result of sympathetic action is secretion of a predominantly low-fluid volume, protein-rich saliva^{125,126} (Table 9-2). Sympathomimetic drugs cause secretion of large amounts of

protein (granular discharge via regulated exocytosis), while the sympatholytic drugs lead to massive accumulation of secretory granules within acinar cells.

Stimulation of parasympathetic nerves releases acetylcholine, which activates the cholinergic receptors, thereby producing a rapid flow of water and ions across the secretory cell into the intercellular canaliculi and lumen.^{125,126} Parasympathetic activation also stimulates cholinergic receptors on the blood vessels of the interstitial tissue, causing vasodilation and increased fluid flow to the secretory endpieces and intercalated ducts. Drugs that mimic the parasympathetic system result in greater salivary flow. Those that act in an opposite manner, such as parasympatholytic agents, block secretion.

In general, the two branches of the autonomic system work in a coordinated manner to regulate secretion. Parasympathetic and sympathetic nerves do not act antagonistically; instead they appear to potentiate each other in producing saliva that contains an appropriate balance of fluid and protein.

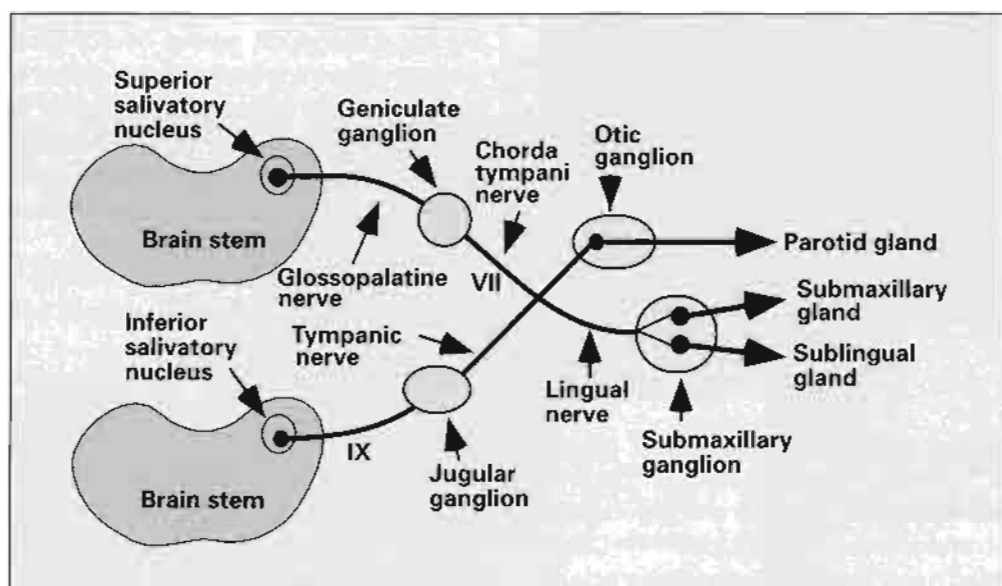


Fig 9-19 Anatomic pathway of the parasympathetic nerve fibers from the brain stem to the major salivary glands. Note the crossover from the superior salivatory nucleus to the submaxillary (submandibular) and sublingual glands, and from the inferior salivatory nucleus to the parotid gland.

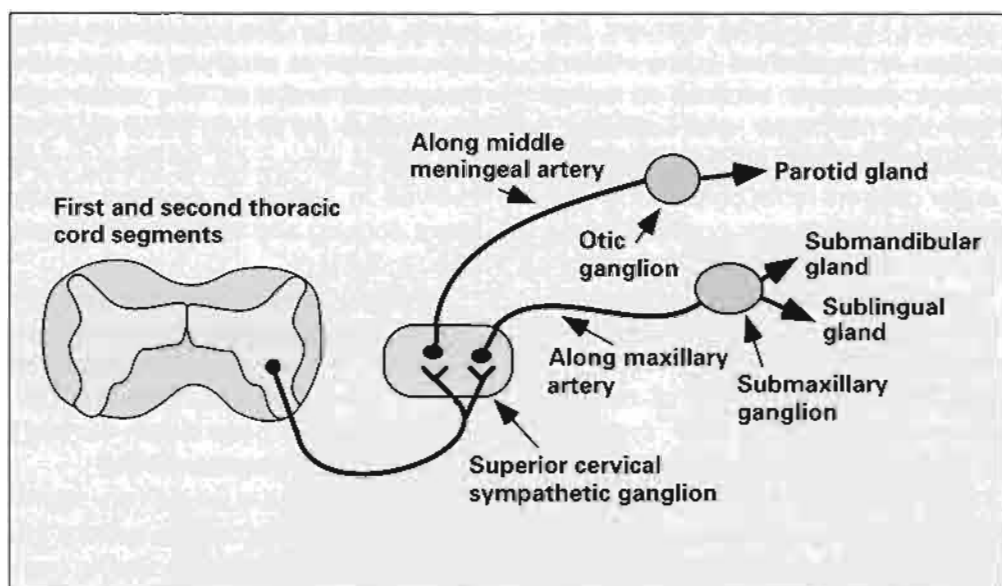


Fig 9-20 Anatomic pathway of the sympathetic nerve fibers from the spinal cord to the major salivary glands. Second-order axons leave the superior cervical sympathetic ganglion, run along the major arteries, pass through the otic and submaxillary ganglion without synapse, and join the parasympathetic fibers in nerves to the individual glands.

In addition to stimulation by adrenergic and cholinergic agents, salivary glands respond to peptidergic neurotransmitters released via reflex pathways that are triggered by somatic afferent inputs. Afferent nerves from pain and touch receptors in the mucosal tissues of the olfactory, masticatory, oral, and pharyngeal surfaces conduct signals for an un-

conditioned reflex stimulation of salivary glands. Experimental evidence shows that nonadrenergic and noncholinergic pathways respond to sensory peripheral input.^{128,129} The nonadrenergic and noncholinergic effector system involves the peptidergic neurotransmitters substance P and vasoactive intestinal peptide. Immunocytochemical studies demonstrate

Table 9-2 General effect of the parasympathetic and sympathetic nerves on the character of salivary output

Type of stimulation	Character of saliva
Parasympathetic (adrenergic)	High fluid volume Low protein
Sympathetic (noradrenergic)	Low fluid volume High protein

vasoactive intestinal polypeptide, calcitonin gene-related peptide, substance P, and neuropeptide Y in nerve fibers associated with mucous and serous acinar cells and components of the ducts.^{130,131} Calcitonin gene-related peptide exerts a vasodilator action on salivary gland blood vessels, thereby increasing blood flow to the glands during salivation.¹³¹

Reflex salivation induced during mastication is a combined result of peptidergic and cholinergic stimulation of acinar cells.¹²⁹ Periodontal ligament mechanoreceptors appear to be involved in the afferent link of the peptidergic response, because consumption of a liquid diet does not trigger reflex salivation.

Secretion of ductal products, such as kallikrein, appears to be under different reflex controls than the secretion of acinar products. Gustatory stimuli cause amylase secretion but no release of kallikrein.

In addition to activating the signal transduction pathways that lead to secretion of saliva, the efferent autonomic nerves also regulate protein synthesis and glandular growth. Repeated exposure of salivary glands to β -adrenergic agonists and sympathomimetic agents causes glandular hyperplasia and increases the formation of secretory granules.¹³² Chronic administration of isoproterenol, a β -adrenergic agonist, increases the expression of cyclin-dependent kinases and their regulatory cyclins in murine salivary glands.¹³³ Destruction of the parasympathetic fibers to the glands leads to glandular atrophy.

Basic Science Correlations

Biochemical events at the synaptic junction

There are two major categories of acetylcholine receptors; one type mimics the action of nicotine and the second type mimics the action of the drug mus-

carine. Nicotinic receptors are present in rapid-acting synapses such as the neuromuscular junction, while muscarinic receptors are found in modulatory synapses such as those that regulate salivary acinar cell activity. In contrast to nicotinic receptors, which trigger rapid responses (contraction of skeletal muscle), the muscarinic receptors activate relatively slow cytoplasmic responses (secretion).

There are at least five muscarinic receptor isoforms. Salivary acinar cells express M1 and M3 isoforms.¹³⁴

Acetylcholine is formed from acetylcoenzyme A and choline by the enzymatic action of choline acetyltransferase (Fig 9-21). Acetylcholine is stored in small, clear synaptic vesicles. Rapid influx of calcium ions through voltage-gated calcium channels opened by the depolarization of the plasma membrane leads to release of neurotransmitters.

Several steps, including protein phosphorylations and actin-vesicle interactions, follow the influx of calcium ions. Calmodulin, a cytoplasmic calcium-binding protein, plays a key role in regulating these events. After binding four calcium ions, calmodulin is then capable of attaching to and activating various cytoplasmic enzymes. The calcium-calmodulin protein kinases are among those enzymes. They play a key role in exocytosis by phosphorylating proteins involved in "priming" synaptic vesicles for subsequent docking and fusion to the plasma membrane (see Fig 9-21).

Following release from synaptic vesicles, acetylcholine diffuses across the narrow synaptic cleft to bind with muscarinic cholinergic receptor in the plasma membrane of the serous acinar cell. Mucous acinar cells have been shown to have both M1 and M3 muscarinic receptors.^{135,136}

Norepinephrine is the primary neurotransmitter of the sympathetic autonomic system. Norepinephrine is synthesized at the nerve ending from tyrosine and stored in synaptic vesicles that have a dense core (Fig 9-22). Docking and fusion of synaptic vesicles (see Fig 9-22 [B and C]) are triggered by entry of calcium ions through voltage-regulated channels opened during membrane depolarization. Norepinephrine binds to α - and β -adrenoceptors in the postsynaptic membrane of the acinar cells. Unbound norepinephrine is taken up along the presynaptic surface (see Fig 9-22 [D]) and deaminated by monoamine oxidase. Extracellular norepinephrine is also converted to inactive normetanephrine by catechol-O-methyltransferase on the postsynaptic plasma membrane.

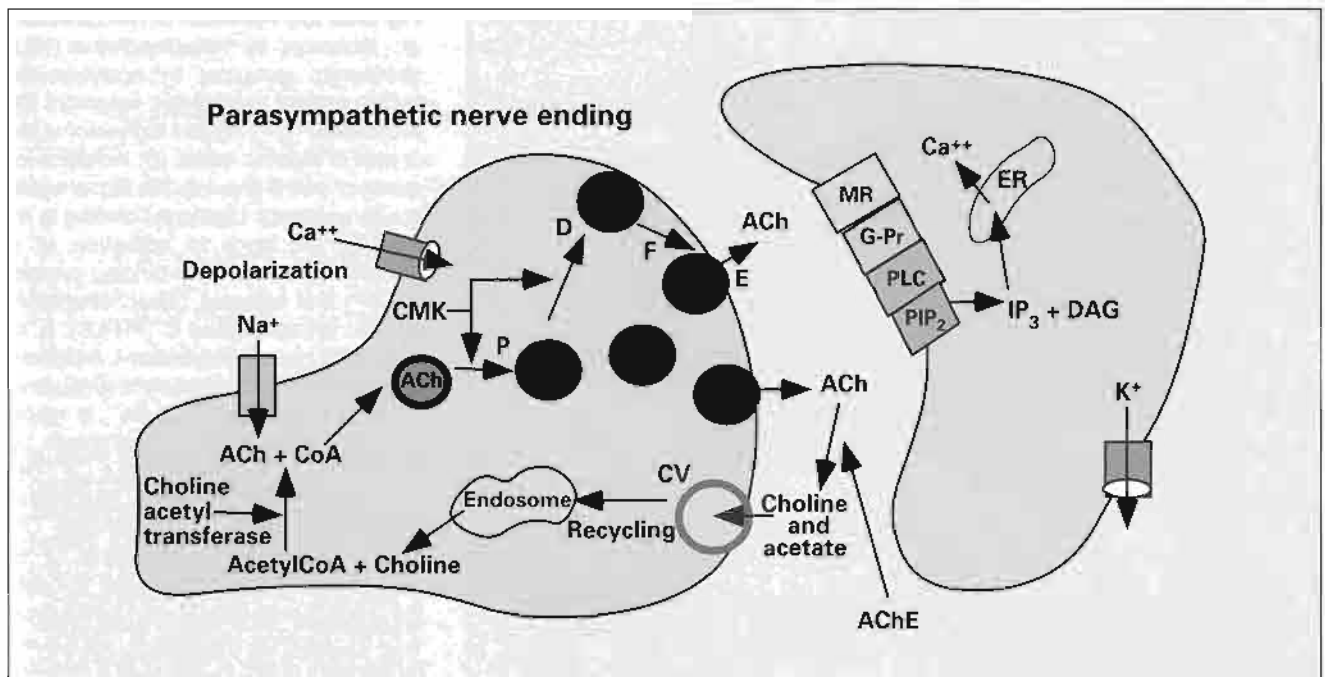


Fig 9-21 Synthesis and packaging of acetylcholine (ACh) neurotransmitter and the steps in its packaging in synaptic vesicles. Voltage-gated calcium channels open following depolarization of the nerve. Calcium ions, interacting with calcium-calmodulin protein kinase (CMK), initiate and control events in the packaging (P), docking (D), and fusion (F) of synaptic vesicles to the plasma membrane. Once released through exocytosis (E), the ACh diffuses in the synaptic cleft and binds to muscarinic receptors (MR) in the salivary acinar cell plasma membrane, leading to the activation of phospholipase C (PLC) and the elevation of cytosolic free calcium. Unbound ACh is cleaved by acetylcholinesterase (AChE) into acetate and choline. Choline is recycled via the endosomal apparatus. (CoA) coenzyme A; (CV) coated vesicle; (G-Pr) trimeric guanosine triphosphate-binding protein; (PIP₂) phosphatidylinositol-4, 5-bisphosphate; (IP₃) inositol triphosphate; (DAG) diacylglycerol; (ER) endoplasmic reticulum.

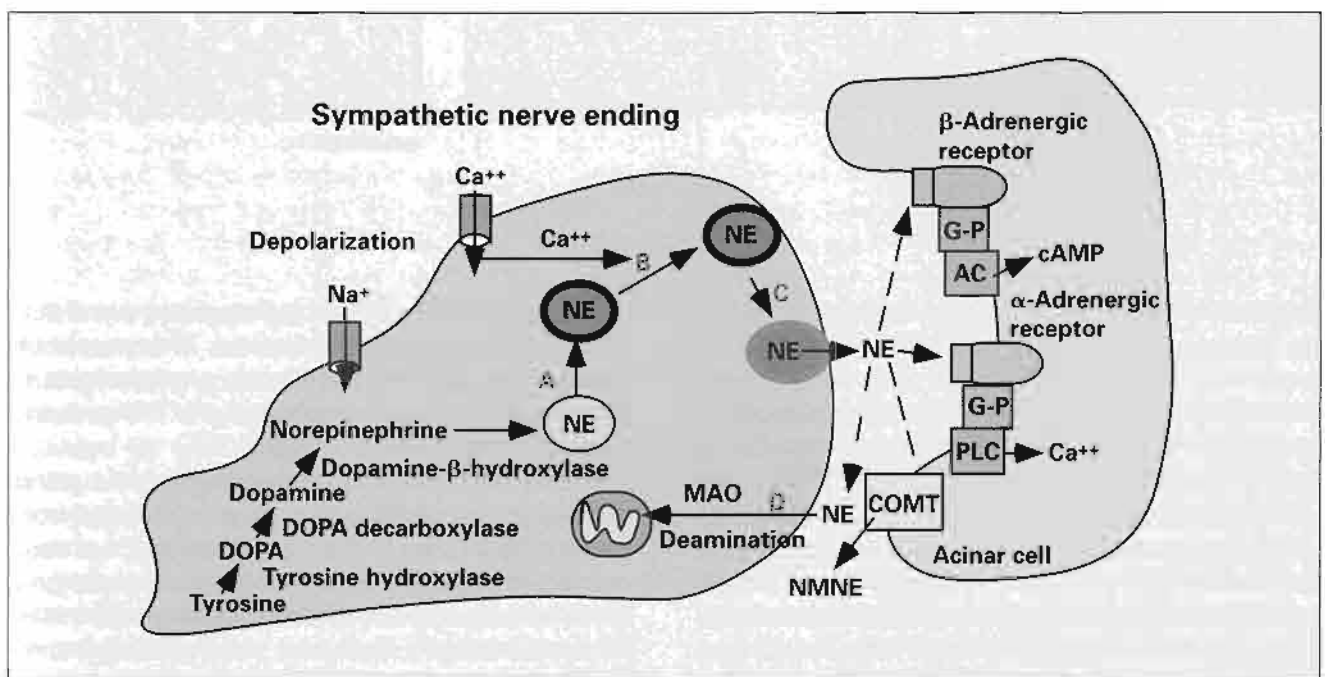


Fig 9-22 Steps in the synthesis and secretion of norepinephrine (NE), the signal transduction pathways triggered by the interaction of NE at β-adrenergic and α-adrenergic receptors in salivary acinar cells, and the metabolism of unbound NE. (A) Transport and storage (blocked by reserpine); (B) priming and docking; (C) exocytosis; (D) uptake and deamination. (AC) Adenylcyclase; (cAMP) cyclic adenosine monophosphate; (COMT) catechol-O-methyltransferase; (DOPA) dihydroxyphenylalanine; (G-P) guanosine triphosphate-binding protein; (MAO) monoamine oxidase; (NMNE) normetanephrine; (PLC) phospholipase C.

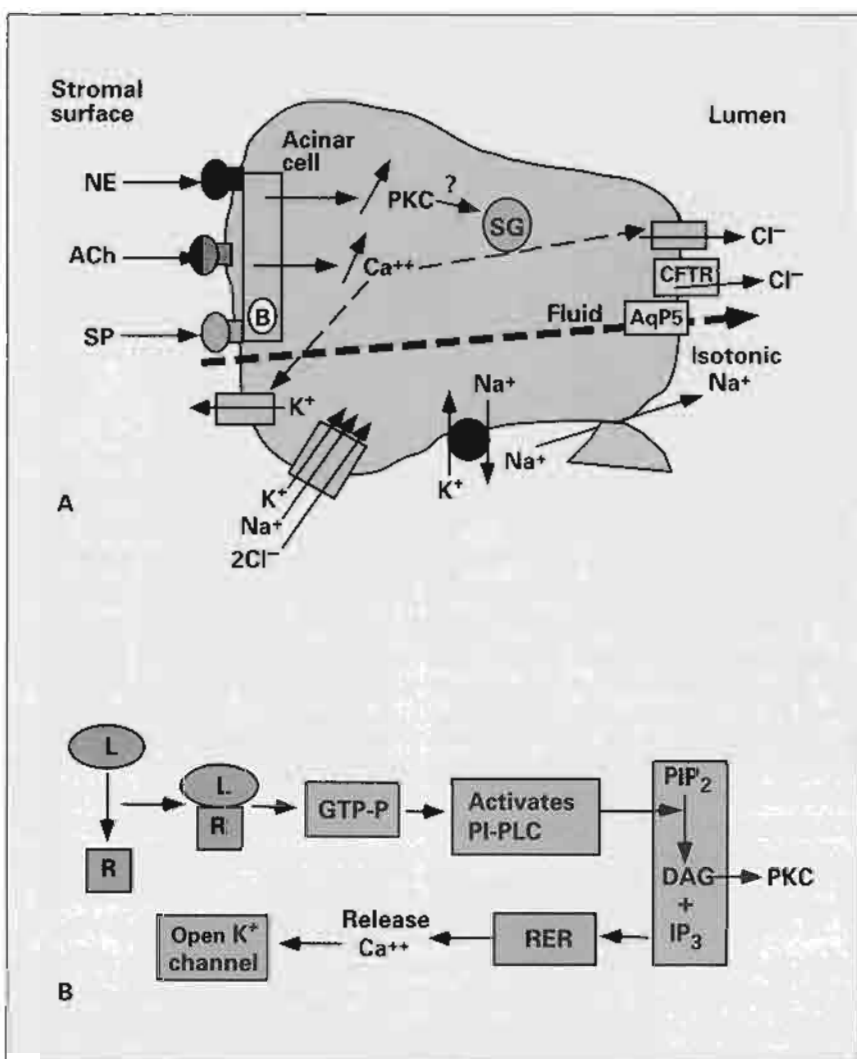


Fig 9-23 (A) Activation of α -noradrenergic receptors by norepinephrine (NE), cholinergic receptors by acetylcholine (ACh), and/or peptidergic receptors by substance P (SP) triggers high-volume secretion of isotonic saliva. **(B)** indicates location of part B drawing. **(B)** Signal transduction pathway. Ligand (L) binding to its receptor (R) leads to activation of a guanosine triphosphate-binding protein (GTP-P) that activates phosphoinositide-specific phospholipase C (PI-PLC). It, in turn, splits phosphatidylinositol-4, 5-bisphosphate (PIP_2) into diacylglycerol (DAG) and inositol triphosphate (IP_3). Ca^{++} is mobilized from intracellular compartments by IP_3 , leading to the opening of a Ca^{++} -dependent K^+ channel in the basolateral membrane and a Ca^{++} -dependent Cl^- channel in the apical membrane. Cl^- is also transported out of the cell through the cystic fibrosis transductance regulator (CFTR). Electrical neutrality is maintained by the entry of Na^+ , K^+ , and Cl^- ions through a cotransporter system in the basolateral membrane. Na^+ ions, pumped into the lateral intercellular space by Na^+ - K^+ -adenosine triphosphatase, diffuse through the "leaky" zonula occludens into the lumen. Along with movement of NaCl , water flows to the lumen through the lateral intercellular spaces and the aquaporins (AQP5) in the apical membrane. The saliva at this point is isotonic. A limited secretory granule (SG) discharge is observed after cholinergic and α -adrenergic stimulation by DAG activation of protein kinase C (PKC). (RER) Rough endoplasmic reticulum.

Signal transduction pathways in acinar cells

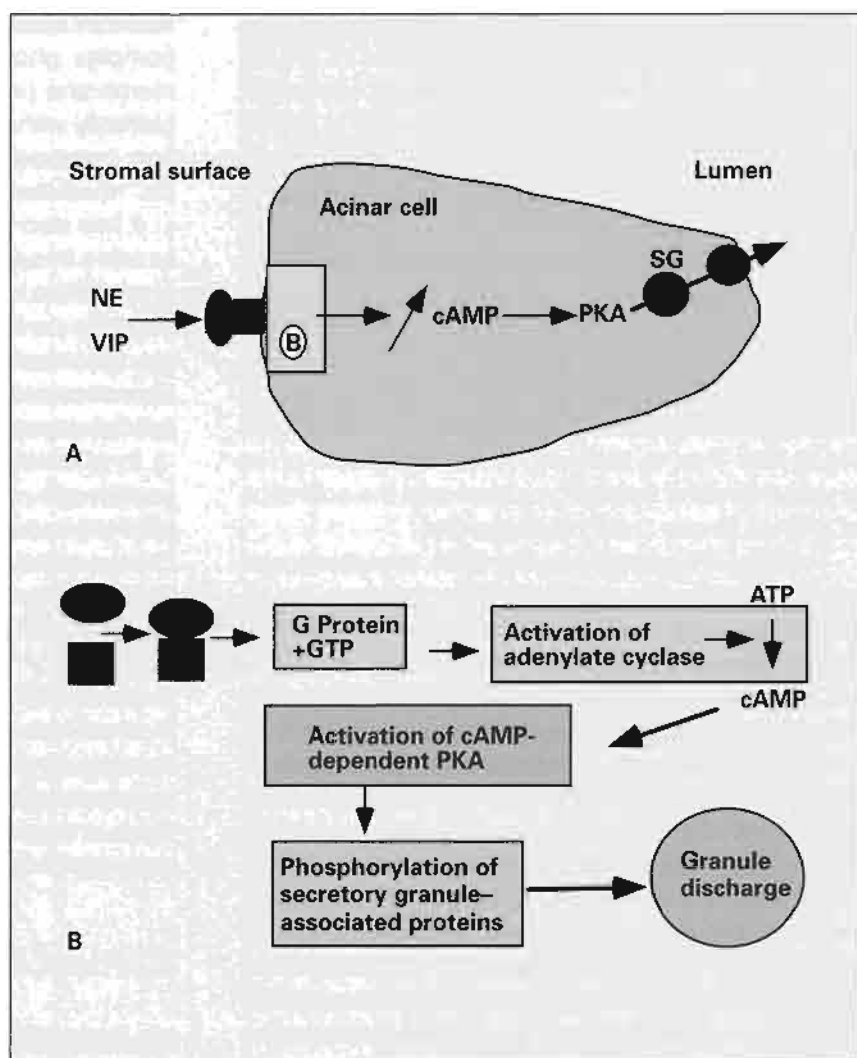
Signal transduction is the process whereby a cell responds to extracellular molecular messengers. These messenger molecules can be neurotransmitters, hormones, growth factors, cytokines, and antigens. The messenger molecule (the ligand) communicates with the interior of the cell by combining with its receptor, usually a transmembrane protein. A conformational change (shape change) in the receptor, induced by the ligand, triggers a cascade of enzymatic reactions.

Each cell type has many different signal transduction pathways, involving hundreds of cytoplasmic proteins and smaller bioactive compounds. Early events in many signal transduction pathways involve

receptor activation of GTP-binding proteins and activation of phospholipase enzymes. The phospholipases attack adjacent membrane phospholipids to generate smaller lipid metabolites that transmit and amplify the receptor-ligand signal.^{44,137}

Neurotransmitter substances released from nerve endings in the salivary gland effect changes in cell function by binding to their receptors in the plasma membrane of the acinar and duct cells. Several signal transduction pathways operate simultaneously in salivary acinar cells.^{102,137} Cellular secretory function results from constant integration and summation of signal transduction events at several metabolic nodal points. These signals activate the cellular mechanisms responsible for secretion of salivary fluids and proteins.

Fig 9-24 (A) Activation of β -adrenergic receptors by norepinephrine (NE) and/or vasoactive intestinal polypeptide (VIP). (B) indicates location of part B drawing. (B) Ligand (L) binding of norepinephrine and its β -adrenergic receptor (R) activates a heterotrimeric guanosine triphosphate (GTP)-binding protein. An activated subunit of the GTP-protein diffuses to and activates the enzyme adenylate cyclase. It generates cyclic adenosine monophosphate (cAMP), which activates a cAMP-dependent phosphokinase A (PKA). PKA phosphorylates cytoplasmic proteins that are believed to have a role in granule transport and secretion. GTP-binding proteins in the granule membrane are phosphorylated by PKA, leading to granule secretion. Binding of VIP to its specific peptidergic receptor triggers the same signal transduction pathway. (ATP) Adenosine triphosphate; (SG) secretory granule.



The two pathways that have been most thoroughly documented in salivary acinar cells are the phosphatidylinositol (calcium) and the cAMP signaling systems.^{102,137} The phosphatidylinositol pathway is activated by cholinergic and peptidergic agonists acting on α -adrenergic, peptidergic, and muscarinic (acetylcholine) receptors (Fig 9-23). The cAMP cascade is triggered by β -adrenergic agonists acting on a β -adrenergic receptor^{137,138} (Fig 9-24).

In the phosphatidylinositol-4, 5-bisphosphate (PIP_2) system (see Fig 9-23), the receptor-ligand interaction activates a GTP-binding protein, which in turn activates phospholipase C.^{102,137,138} This enzyme cleaves PIP_2 into diacylglycerol and inositol triphosphate. Both of these substances act as second messengers within the cytoplasm. Elevation of diacylglycerol fol-

lowing cholinergic stimulation is known to activate protein kinase C.¹³⁹ The latter enzyme may be involved in amylase secretion (granular discharge) from parotid glands.^{139,140}

Inositol triphosphate interacts with the membranes of the RER or other cytoplasmic compartments (calciosomes) to release calcium into the cytoplasm.¹⁰² In turn, the increased cytosolic calcium opens calcium-dependent potassium channels in the basolateral plasma membrane, allowing K^+ to escape and Na^+ and Cl^- to enter the cell through a cotransporter mechanism.¹⁴¹ Calcium ions also open a calcium-dependent chloride channel in the apical membrane.¹⁴² Furthermore, Ca^{++} induces the translocation of aquaporin 5 from the cytoplasm to the luminal membrane.⁶⁶ Excess sodium is actively pumped out

of the cell via the $\text{Na}^+\text{-K}^+\text{-ATPase}$, also located in the basolateral plasma membrane.

Recent studies have shown that zonula occludens junctions are dynamic structures that open to permit ion flow through a paracellular route in response to nerve stimulation. Sodium ions diffuse down a concentration gradient across the "leaky" zonula occludens to gain access to the lumen. Fluid movement across the cell, and/or through the intercellular spaces into the lumen, accompanies the transepithelial movement of Na^+ and Cl^- .

A small amount of granule exocytosis accompanies α -adrenergic and peptidergic stimulation (see Fig 9-23). This is believed to be the result of increased levels of cytosolic calcium and the activation of protein kinase C via diacylglycerol.

In the cAMP system (see Fig 9-24), receptor-ligand binding produces a conformational change in the receptor, causing it to interact with a heterotrimeric GTP-binding protein. As a result of this interaction, the α subunit of the GTP-binding protein binds GTP and releases from the parent molecule. The α subunit binds with and activates a nearby molecule of adenylate cyclase. All of these reactions occur at the plasma membrane.

The activated adenylate cyclase converts ATP to cAMP (the second messenger). Cyclic adenosine monophosphate initiates a cascade of cytoplasmic events via its ability to activate cAMP-dependent protein kinase A. The latter enzyme is responsible for the phosphorylation of serine and/or threonine residues of other cytoplasmic proteins, some of which are believed to have a role in secretory granule exocytosis.⁵² In addition, protein kinase A phosphorylates cAMP response element-binding protein, a transcription factor involved in regulating the production of mRNAs for several salivary proteins.^{52,143}

The precise role of protein kinases and their protein substrates in the secretory process has yet to be fully understood. Proteins contained in the granule membrane are also involved in regulating secretion. Vesicle-associated membrane proteins (VAMPs or SNAPs) that anchor synaptic vesicles to the plasma membrane at axon terminals have been localized on salivary acinar cell granules and have been shown to be required for cAMP-mediated granular discharge.⁴⁷ Members of the cytoplasmic family of monomeric GTP-binding proteins, with known functions in vesicular trafficking, have also been localized in the secretory granule membrane.¹⁴⁴ Because they can be activated (phosphorylated) by protein kinase A, they are suspected to regulate granular discharge. Addi-

tional studies have shown that the Ca^{2+} -calmodulin complex phosphorylates several secretory granule membrane proteins. The cAMP pathway acts synergistically with the PIP_2 pathway during salivary secretion because of the ability of cAMP to potentiate Ca^{2+} -mediated responses.¹⁴⁵

It has also been shown that dephosphorylation of tyrosine by specific protein tyrosine phosphatase is a prerequisite to secretory granule fusion and exocytosis in the parotid acinar cells of rats.¹⁴⁶ It appears that granular secretion involves a balance and/or proper sequence of both phosphorylation and dephosphorylation events.

In addition to the adrenergic, cholinergic, and peptidergic receptor-initiated responses, purinergic (ATP), 5-hydroxytryptamine, and neuropeptide Y receptor-initiated secretory responses have been demonstrated in rat salivary glands.^{137,147-149} Activation of these receptors modulates the secretion resulting from stimulation of the major parasympathetic and sympathetic pathways.

Most of the current knowledge of signal transduction in salivary secretion comes from studies in experimental animals. These studies have revealed that there are many interspecies differences in innervation, signaling pathways, and transport systems.

Clinical Correlations

Xerostomia (dry mouth)

Xerostomia is a debilitating condition that, in its most severe form, leads to numerous secondary disorders of the oral tissues.^{150,151} Numerous elderly patients experience chronic dry mouth; women report this chief complaint at a much higher rate than do men.^{150,151} Reduced salivary secretion can be a component of a systemic disease affecting many organ systems of the body or it can be a local disorder involving only the salivary glands.^{150,151}

The cause of the problem can be organic, as in destruction of glandular secretory cells by inflammation or by radiation to the gland, or it can result from functional alteration of otherwise normal secretory cells, as in the side effects of medications that interfere with the neuroregulation of secretion. In the case of an organic problem, a substantial portion of the salivary tissue must be destroyed before dryness becomes problematic. Studies have shown that 50% to 75% of the glands must be nonfunctional before salivary flow is reduced to the level that gives rise to the sensation of dry mouth.

Chronic deficiency of salivary secretion has several negative consequences^{150,152}:

1. Increased incidence of dental caries
2. Atrophy of mucosal surfaces
3. Greater chance for oral infections
4. Difficulty with swallowing
5. Burning sensation affecting the tongue
6. Altered taste perception

Xerostomia is often a result of systemically administered medications that target autonomic neurotransmitter–ligand interactions. Drugs that block muscarinic cholinergic receptors, and α - and β -adrenergic receptors impair salivary secretion (Table 9-3). More than 400 drugs have been shown to be responsible for the side effect of dry mouth.¹⁵³ Among the most cited medications are tricyclic antidepressants and the phenothiazine antipsychotics that block α -adrenergic receptors.^{153,154} Anticholinergic drugs used in the treatment of Parkinson's disease and peptic ulcers also reduce salivary flow by blocking muscarinic acetylcholine receptors. Drugs that block β -adrenergic receptors decrease granular discharge and therefore decrease the salivary concentration of protective substances, such as histatins.

Sjögren's syndrome

The attributes of self and nonself (foreignness) are monitored by a surveillance of membrane proteins displayed on the major histocompatibility complex at the cell surface (see chapter 13). Autoimmune diseases arise when normal cells are wrongly identified as foreign and are targeted for destruction by cytotoxic lymphocytes or when components of the extracellular tissues that have been altered during chronic inflammation trigger the formation of autoantibodies and subsequent tissue destruction by mononuclear phagocytic cells.

Sjögren's syndrome is a systemic autoimmune disease involving the salivary glands and the lacrimal glands.^{151,155–157} Antibodies reacting with salivary gland duct epithelium are present in the serum of patients with Sjögren's syndrome.¹⁵⁸ The salivary glands in people with Sjögren's syndrome are characterized by periductal lymphocytic infiltration, acinar and ductal cell death, and reduced salivary output.^{151,156,157,159–161} The periductal lymphoid tissue contains germinal center structures rich in B cells.¹⁶⁰ Parenchymal cell death occurs by Fas/FasL-mediated apoptosis¹⁶² (see chapter 13).

Table 9-3 Drugs that decrease salivation

Drug category	Receptor block
Antipsychotics	α -Adrenergic
Antidepressants	α -Adrenergic
Antiparkinsonisms	Cholinergic
Antihypertensives	β -Adrenergic
Antihistamines	Cholinergic
Anticholinergics	Cholinergic

Patients afflicted with this disorder experience dry and painful oral mucosal surfaces, difficulty with swallowing, increased incidence of oral infections, and an increased incidence of dental caries.^{151,156} Sjögren's syndrome affects primarily middle-aged women. It has been estimated that approximately 1 of every 1,000 individuals is affected with this problem.

Despite many studies of this disease, its specific cause is not understood.¹⁵⁶ It has been suggested that tissue alteration, perhaps induced during a viral infection of the glandular tissue, may lead to the expression and display of "foreign" molecular species at the cell surface of salivary epithelial cells, triggering an immune response. Infiltration of the glands by lymphocytes would then gradually destroy and replace the acinar secretory units.

Recent findings of the presence of Epstein-Barr viral DNA and viral products in the epithelial cells and lymphocytes of salivary glands from patients with Sjögren's syndrome suggest that this disease may indeed have a viral etiology.^{156,163} Epithelial cells that contain viral products are targets for cytotoxic T cells. Chronic presence of viral genomic material in lymphocytes could lead to continued proliferation and activation of B and T cells in the glandular tissues and elevated local expression of inflammatory cytokines.

Increased production of IL-2, IL-6, interferon γ , and transforming growth factor β has been detected by amplification of mRNA in the epithelial cells and infiltrating lymphocytes of the salivary glands of patients with Sjögren's syndrome.^{164,165} The report of increased expression of the secretory component of IgA and major histocompatibility complex II protein

in the salivary epithelial cells of Sjögren's patients is consistent with increased local production of interleukins.¹⁶⁶ A high percentage of salivary gland epithelial cells in patients affected by Sjögren's syndrome show increased expression of secretory component (polymeric IgA receptor).

Immunocytochemical studies of salivary glands in individuals with Sjögren's syndrome have also revealed a disturbance in anion exchange ($H^+HCO_3^-$) in the striated ducts.¹⁶⁷ Decreased extrusion of H^+ leads to elevated intracellular pH and cellular damage. It is not clear if the decrease in anion exchanger is secondary to inflammation or whether it is a component of the primary lesion. Abnormalities in the protein content of the saliva of patients with Sjögren's syndrome have also been reported.¹⁶⁷

Several animal models of Sjögren's syndrome are currently under investigation. The nonobese diabetic mouse appears to be a good animal model of Sjögren's syndrome.¹⁶⁸ Nonobese diabetic mice develop lymphocytic infiltration of the submandibular and lacrimal glands and demonstrate reduced secretion of saliva and tears. Of interest is the finding that the salivary glands of nonobese diabetic mice have reduced levels of β -adrenergic receptors and adenylyl cyclase.¹⁶⁹ It has been suggested that the sera of nonobese diabetic mice contain antibodies directed to the β -adrenergic receptor. These results point to a defect in signal transduction as a possible cause of reduced salivation in Sjögren's syndrome.

The MRL/lpr mouse strain is another model of this disease. The salivary glands of these mice have high levels of IL-12 and interferon γ .¹⁷⁰ Both of these cytokines stimulate the expression of major histocompatibility complex II molecules and the production of antibodies. Macrophages, CD4⁺ lymphocytes, and B cells are present in high numbers in the periductal connective tissue of the MRL/lpr mouse salivary glands.

Of additional interest is the observation that normal mice injected intraperitoneally with IL-2 rapidly develop lymphocytic infiltration of salivary glands and decreased salivary output. Cessation of IL-2 administration results in full recovery within a few days. These results suggest that secretion of IL-2 by salivary lymphocytes might have a toxic effect on acinar cells in Sjögren's syndrome. Although it is reasonable to expect a lymphokine effect in Sjögren's syndrome, it does not explain the primary cause of the lymphocytic infiltration.

Gene transfer therapy potential of the salivary glands

The ability to access the salivary gland parenchyma via retrograde transport has led to research on the use of salivary acinar cells as mediators in gene therapy. Baum and coworkers^{65,171-173} have pioneered the introduction of genes into salivary glands via retrograde infusion of cationic lipoplexes containing plasmid DNA expression vectors. Encouraging results have shown that a small but reproducible percentage of acinar cells respond by expressing the protein encoded by the newly introduced DNA.

Rat submandibular glands expressed human growth hormone and anti-influenza immunoglobulins following the instillation of DNA encoding human growth hormone and influenza protein, respectively.^{65,172} Although human growth hormone was secreted into saliva at a low concentration (10.8 ng/mL), it was suggested that this level, depending on the specific protein, might be sufficient to exert a therapeutic effect.

Results have demonstrated the potential of salivary gland gene therapy for the treatment of various diseases not otherwise treatable by conventional means.

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Chapter 10

Oral Somatosensory Systems



The mouth is richly innervated with a variety of sensory end-organs or receptors. A large area of the cerebral cortex receives input from sensory end-organs in the tissues of the face and oral cavity. Figure 10-1 shows the relative amounts of somatosensory cortex devoted to each region of the body.¹ The lips, teeth, gingiva, and tongue account for an area equal in size to that of the hand.

Somatosensory afferents from the oral mucosa coordinate a variety of oral functions. The greatest concentrations of nociceptors (pain receptors) and mechanoreceptors are found in the anterior parts of the gingiva, tongue, and hard palate. The rich innervation of the anterior part of the hard palate is important in the production of speech. Nerve endings in the gingiva and palate help to coordinate the movement of food posteriorly during chewing. Mechanoreceptors in the periosteum of the jaws and in the periodontal ligaments (PDLs) influence muscle activity during the mastication of food.

Types of Cutaneous Somatosensory Receptors

Before the structure and function of oral sensory receptors are discussed, the current knowledge of cutaneous receptors and the nerve fibers that conduct afferent stimuli must be reviewed. Cutaneous sen-

sory receptors fall into three major types: mechanoreceptors, nociceptors, and thermoreceptors. Peripheral nerve fibers are classified on the basis of fiber diameter and conduction velocity (Table 10-1). Larger diameter fibers conduct at higher speeds. This discussion is concerned primarily with A β , A δ , and C fibers that are involved with the peripheral somatosensory system.

Mechanoreceptors of glabrous (nonhairy) skin

Four classes of mechanoreceptors have been identified, based on their electrophysiologic responses and anatomic structure.² Types 1 through 4 are derived from group II or A β fibers, ie, 6 to 12 μ m in diameter and having a conducting velocity of about 35 to 70 m/s.

Type 1: Rapidly adapting receptor II (RA-II)

This receptor has a large receptive field with poorly defined boundaries. It is a rapidly adapting detector of tissue vibration and deformation. The Pacinian corpuscle is an RA-II.

Type 2: Rapidly adapting receptor I (RA-I)

This is a rapidly adapting mechanoreceptor with a small receptive field, responding to tissue movement

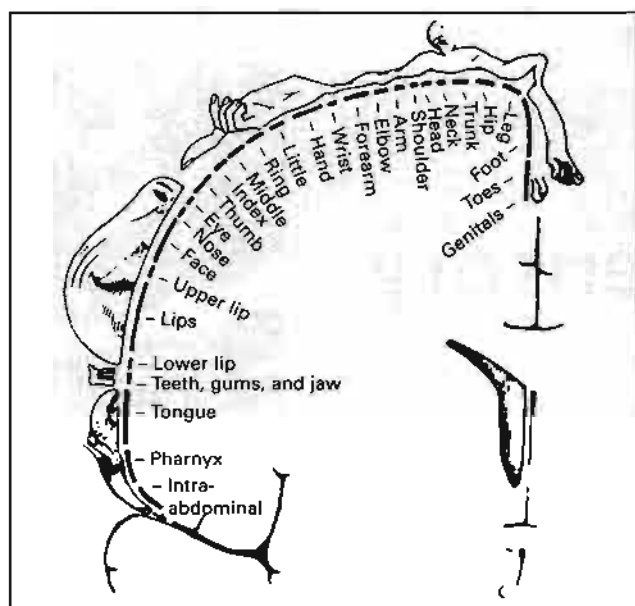


Fig 10-1 Relative area of the brain cortex receiving input from peripheral somatosensory receptors. Note the large area devoted to the orofacial region. (Reprinted from Penfield and Rasmussen¹ with permission from The Gale Group.)

Table 10-1 Classification of peripheral nerve fibers

	Fiber type	Diameter (μm)	Conduction velocity (m/s)	Source
I	A α	12.0–21.0	70.0–120.0	Muscle spindles Golgi tendon organs Motoneurons to spindles
II	A β	6.0–12.0	35.0–70.0	Muscle spindles
	A γ	2.0–8.0	12.0–48.0	Low-threshold mechanoreceptors Motoneurons to spindles
III	A δ	1.0–6.0	2.5–35.0	Low-threshold mechanoreceptors Thermoreceptors Nociceptors Preganglionic autonomic nerves
IV	B	1.0–3.0	2.5–15.0	Low-threshold mechanoreceptors
	C	0.4–1.2	0.7–1.5	Thermoreceptors Nociceptors Postganglionic autonomic fibers

characterized as low-frequency vibration.² The morphology of the RA mechanoreceptor consists of a simple encapsulated ending. Meissner's corpuscle is an example of an RA-I.

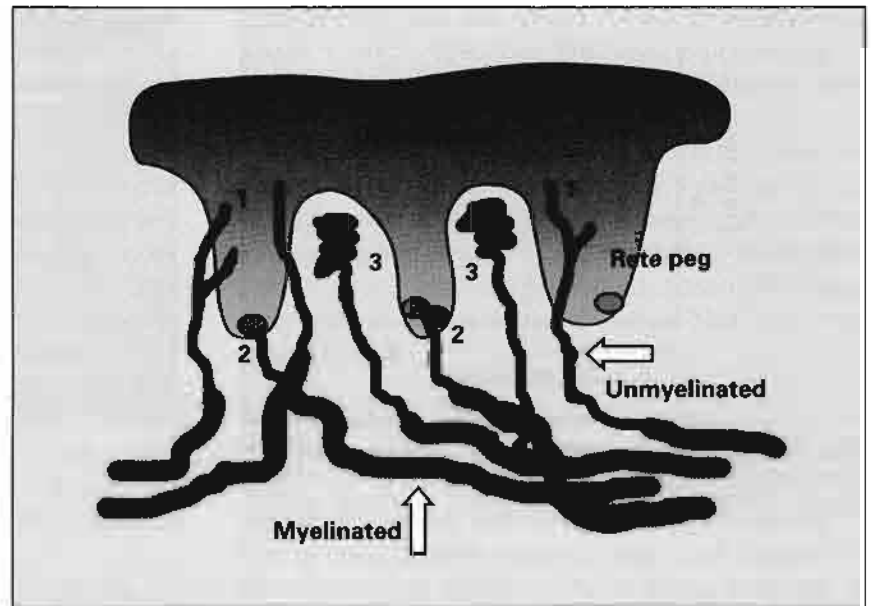
Type 3: Slowly adapting receptor I (SA-I)

A small receptive field responding to sustained indentation of adjacent tissues characterizes the SA-I receptor.² It discharges irregularly throughout stimulus application. It provides for two-point discrimination and spatial detail. The Merkel cell-neurite complex is an SA-I.

Type 4: Slowly adapting receptor II (SA-II)

This type of receptor responds to static and stretch displacement of adjacent tissues over a large receptive field with poorly defined boundaries. A regular discharge is produced throughout stimulus application. These receptors are richly distributed in the skin over joints and in ligaments, serving to provide proprioceptive (position and motion) input to spinal and higher centers. The Ruffini-type nerve ending and its associated collagenous component is thought to be the anatomic structure responsible for SA-II somatosensory input.

Fig 10-2 Nerve supply to the oral mucosa. The subepithelial nerve plexus gives rise to parent nerve fibers for three types of end-organs: (1) free nerve endings; (2) Merkel cell-neurite complexes; (3) encapsulated receptors resembling Meissner's corpuscles.



C mechanoreceptors

C mechanoreceptors respond at very low threshold levels to slow brushing, scratching, and stretching movements of the skin. They fire at the onset of stimulus application and show after-discharge following stimulus removal. Repeated stimulation leads to fatigue or desensitization of the receptors. Sensory input to *C* mechanoreceptors originates from free nerve endings.

Cutaneous A δ and C nociceptors

These free nerve endings respond to noxious mechanical, thermal, and chemical stimulation. They have a relatively small receptive field and higher thresholds for mechanical and thermal stimulation than do simple *C* mechanoreceptors and *C* thermal receptors.

Electrophysiologic studies show that many receptors respond to a variety of stimuli. Polymodal nociceptors respond to thermal, chemical, and mechanical stimulation but produce a sensation of pain when stimulated at higher intensities. Most mechanoreceptors provide a stronger response to a specific type of stimulation; for example, fast-rate, rapidly adapting responses are primarily associated with encapsulated nerve terminals.²

Innervation of the Oral Mucosa

Sensory nerve plexus

The nerve supply to the oral mucosa consists of a nerve plexus and its terminal end-organs (Fig 10-2). The nerve plexus is a complex network of myelinated and unmyelinated fibers present in the connective tissues of the oral mucosa.³ The network is subdivided into a deep submucosal plexus, consisting of larger myelinated nerve bundles, and a superficial subepithelial plexus consisting of fine myelinated and unmyelinated nerve fibers. These fibers terminate as receptors within the epithelium and in the dermal papillae. The fibers of the deep plexus connect and give rise to the superficial plexus and innervate deeper structures of the submucosa, such as the minor salivary glands.

The nerve plexus has a complex and highly interdigitated organization. Its greatest density is in the anterior parts of the mouth, especially the dorsum of the tongue, the palate, and the gingiva.³ In general, the density of innervation parallels the density of dermal papillae. The fibers that contribute to the sensory terminals or receptors are arranged so that each fiber innervates a localized area of the mucosa, thereby permitting accurate discrimination of spatially related sensory stimuli.

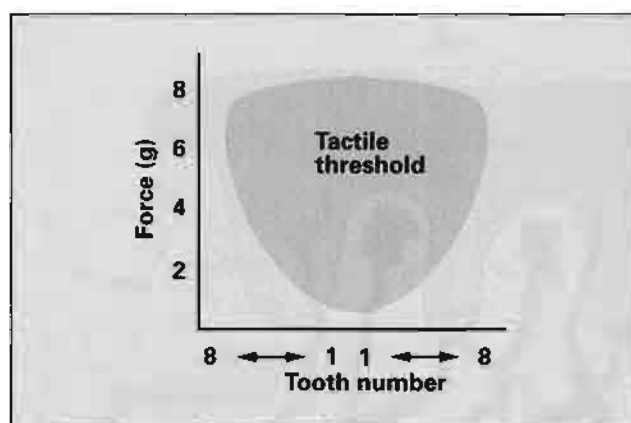


Fig 10-3 Relationship between tooth position and tactile threshold. Greater force is needed to obtain a positive tactile response from the posterior teeth. (Adapted from Manly et al.⁵)

Touch (tactile) sensation is most highly developed in the anterior parts of the oral cavity. The tactile threshold for teeth varies from 1 to 10 g and is lowest in the anterior teeth (Fig 10-3).^{4,5} Measurements of two-point discrimination indicate the richness of somatosensory innervation. This is a measure of the minimal distance between two points at which they are felt as separate points. Two-point discrimination is 1.7 mm on the tip of the tongue and 2.4 mm on the upper lip (Table 10-2).⁶ The amount of mechanical stimulation needed to elicit a response is lower on the tip of the tongue and lips than it is on most other surfaces of the body.

Receptor end-organs are the specialized terminal elements of the nerve plexus that act to transduce physical and chemical stimuli into electrical impulses of nerve transmission.

Terminal end-organs

Three types of somatosensory receptors are located in oral mucosa: (1) free nerve endings (pain and temperature), (2) the Merkel cell-neurite complex (slowly adapting mechanoreceptor), and (3) organized, encapsulated receptors resembling Meissner's corpuscles of the skin (rapidly adapting mechanoreceptors). Deeper tissues of the mouth contain Ruffini nerve endings, pacinian corpuscles, Golgi tendon organs, simple encapsulated receptors, and free nerve endings.

Free nerve endings (nociceptors)

The free nerve ending is the most numerous type of nerve termination. Free nerve endings are axon ter-

Table 10-2 Two-point discrimination

Body part	Discrimination
Tip of tongue	1.7 mm
Upper lip	2.4 mm
Mandible	5.5 mm
Palm	7.5 mm
Forehead	8.8 mm
Back of hand	11.8 mm

Based on data from Sato et al.⁶

minals devoid of myelin and Schwann-cell covering. It has long been accepted that sensory free nerve endings mediate the sensation of pain in response to noxious thermal, chemical, and mechanical stimuli. In the oral mucosa, free sensory nerve endings are found in the epithelium, the lamina propria, and the submucosal connective tissues. Basal lamina material partially covers free nerve endings in the connective tissues but is completely absent over nerve endings that terminate in the epithelium. Several free nerve endings may derive from unmyelinated branches of a single unmyelinated C fiber or from a single, thinly myelinated A δ fiber. Free nerve endings provide an extensive neural receptive surface next to and inside the epithelium.

When mechanical, thermal, and chemical stimuli reach threshold intensities, they activate C- and A δ -nociceptive free nerve endings. Stimulation of A δ -fiber free nerve endings results in sharp pain (first pain), while the activation of C-fiber free nerve endings gives rise to a dull, longer lasting pain that is a poorly localized, burning sensation. The long-lasting and diffuse nature of the pain associated with C-fiber nociception is in part the result of the extensive interneuronal connections of C fibers within the brain stem and the local release of neuropeptides (see Perl's review⁷ of the early research to characterize nociceptors). The C-fiber nociceptors are not involved in precise localization or in two-point discrimination.

Two major classes of C-fiber nociceptor neurons have been identified on the basis of differences in growth factor dependence, neuropeptide content, cell membrane receptors, and their anatomic loca-

tion within the dorsal root ganglion.^{8,9} Nerve growth factor (NGF)-dependent neurons are located in the outer layer (lamina I) of the dorsal root ganglion. These nociceptors contain substance P (SP) and calcitonin gene-related peptide (CGRP) and are implicated in the hyperalgesia associated with inflammation. Nerve growth factor-dependent neurons express the vallinoid receptor (VR1), a key responder to noxious heat and acid pH, modulated by capsaicin. Nociceptors of the second type are dependent on glial cell-derived neurotrophic factor and are located in deeper layers (lamina II) of the dorsal root ganglion.⁹ Glial cell-derived neurotrophic factor-dependent nociceptors express high levels of an ionotropic receptor for adenosine triphosphate, the P2X3 protein. Adenosine triphosphate produces pain when applied to nerve endings.

Great progress has been made in identifying the cell membrane receptors responsible for detecting noxious stimuli. Identification of VR1 represented a milestone in the biology of pain physiology.¹⁰ Vallinoid receptor gets its name from the vallinoid group of capsaicin, a compound responsible for the burning sensation produced by "hot" peppers. A chemically gated six-pass transmembrane protein channel, VR1 favors Ca^{++} permeation. Channel opening occurs in response to capsaicin, acid, and heat greater than 43°C. Both C and A δ nociceptors express VR1. The receptor is of special significance in responding to rapid increases in temperature and to increased tissue acidity generated in ischemic and inflammatory conditions.¹¹⁻¹⁴ In addition, VR1 is also subject to sensitization through second messenger systems activated by various inflammatory mediators.^{10,15}

A vallinoid-like receptor is capsaicin resistant and heat sensitive at temperatures greater than 52°C. The vallinoid-like receptor is expressed in medium- to large-diameter sensory neurons supplying peripheral tissues, including the dental pulp.¹⁶

Other plasma membrane channel proteins with a role in activating nociceptors include acid-sensing ion channels, tetrodotoxin-resistant voltage-gated sodium channels, and the adenosine triphosphate ionotropic P2X3 receptor.^{14,16,17} Acid-sensing ion channels and adenosine triphosphate receptors are thought to have special significance in pain originating from inflammation. Acid generated during ischemia and adenosine triphosphate released from dying cells are components of the inflammatory process.

All of the aforementioned channel proteins can be modulated by a variety of inflammatory signaling molecules. The reader interested in inflammation-induced

pain should read the recent review by Scholz and Woolf,¹⁵ which describes the many components of the "inflammatory soup" and how they interact with nociceptor nerve endings.

In addition to its nociceptive functions, the free nerve ending may act as an effector end-organ by releasing neuropeptides when repeatedly stimulated or when hypersensitized (see "Biology of nociceptor sensitization," later in the chapter). These neuropeptides act on adjacent blood vessels and inflammatory cells.

Merkel cell-neurite complex

The Merkel cell is a specialized epidermal cell that forms part of the diffuse neuroendocrine system.¹⁸ In the mouth, Merkel cells are preferentially located in the gingiva, buccal mucosa, and hard palate.¹⁹⁻²¹ Identification of Merkel cells in tissue sections is aided by staining with antibodies to keratins 18, 19, and 20; to chromogranin A, a matrix component of the Merkel cell granules; and to neural cell adhesion molecule.^{22,23} Merkel cells are found in the basal and spinous layers of the epithelium, concentrated at the base of rete pegs (Figs 10-4a and 10-4b).²⁴⁻²⁶ They develop a close contact to intraepithelial nerve endings to form the Merkel cell-neurite complex. The complex functions as a slowly adapting mechanoreceptor with a relatively small receptive field (type SA-I).

Merkel cells and their associated nerve endings express receptors for, but do not make, epidermal growth factor. On the other hand, epidermal growth factor is expressed by the adjacent keratinocytes, suggesting that they control the differentiation of the Merkel cell via a paracrine epidermal growth factor effect. The survival of the mature Merkel cell is also dependent on neurotrophic factors secreted from the associated nerve ending. Severance of the A β fibers supplying the Merkel cell-neurite complex leads to atrophy of the Merkel cells.

There is also evidence of reciprocal stimulation. Merkel cells may act as target cells, secreting substances that attract afferent sensory axons toward their location in the epithelium.²⁷

The Merkel cell contains many densely stained granules, 50 to 140 nm in diameter, surrounded by a limiting membrane (Figs 10-5 and 10-6).²⁴ Most granules are concentrated between the Golgi complex and the plasma membrane that abuts the adjacent nerve terminal. These granules originate from the Golgi apparatus and are secretory in nature. Neuropeptides (histidine isoleucine, vasoactive intestinal polypeptide, CGRP, and SP) have been localized in Merkel cell granules by immunocytochemistry.

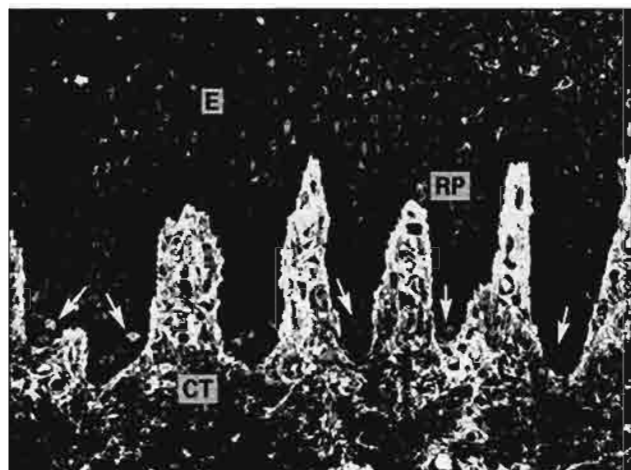


Fig 10-4a Location of Merkel cells (arrows) in the base of the epithelial rete pegs (RP) of the palate. (E) Epithelium; (CT) connective tissue. (Original magnification $\times 200$.)

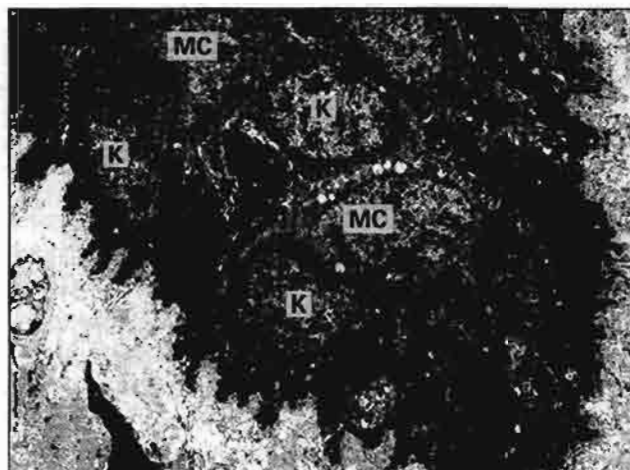


Fig 10-4b Base of a rete peg containing several Merkel cells (MC). (K) Keratinocyte. (Original magnification $\times 3,400$.)

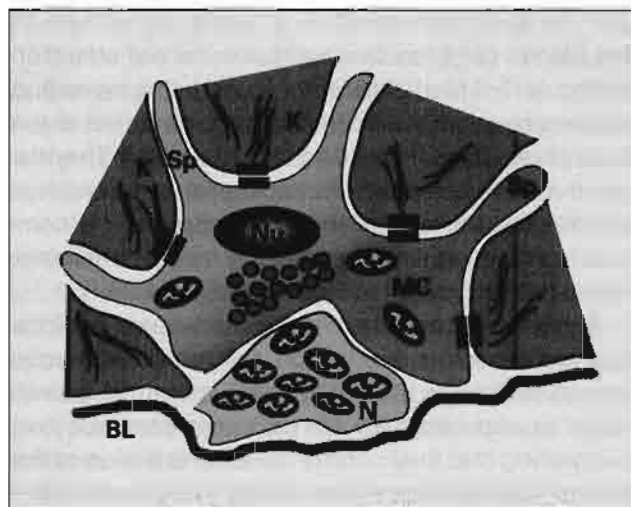


Fig 10-5 Structure of the Merkel cell. The Merkel cell (MC) and its neurite (N) are situated between the basal lamina (BL) and the epithelial keratinocytes (K). Electron-dense granules are concentrated between the nucleus (Nu) and the adjacent neurite. Keratinocytes and Merkel cells are attached by desmosomes. Cytoplasmic processes, or spines (Sp), project from the MC into the intercellular spaces between keratinocytes.

The Merkel cell plasma membrane facing the nerve ending appears to have a synaptic specialization. Experiments have shown that depletion of Merkel cell granules leads to a decreased responsiveness of the neurite. It has been suggested that the release of the neuropeptides in the immediate vicinity of the nerve ending keeps the receptor in a heightened state of responsiveness. The Merkel cell neurite contains numerous mitochondria, indicating that it has a high metabolic activity.

Merkel cells have many spiny cytoplasmic processes that project into adjacent intercellular spaces and indent the cytoplasm of adjacent keratinocytes (see Figs 10-5 and 10-6a).²⁴ These rigid spines con-

tain cytoplasmic filaments that interconnect with larger filament networks in the Merkel cell cytoplasm. The function of these spiny projections is unclear. However, they appear suited for detecting and amplifying the movement of the adjacent epithelium, possibly by deforming stretch-activated cation channels.²⁸ It has been speculated that mechanical displacement of the epithelium, amplified by the spines of the Merkel cell, leads to discharge of neurotransmitter granules and activation of the adjacent nerve ending.

Voltage-gated calcium channels have been demonstrated in Merkel cells.²⁹ It has been suggested that increased intracellular calcium controls the release



Fig 10-6a Characteristic features of Merkel cells (MC). (K) Keratinocytes; (S) cytoplasmic spines of the Merkel cell. (Original magnification $\times 12,600$.)

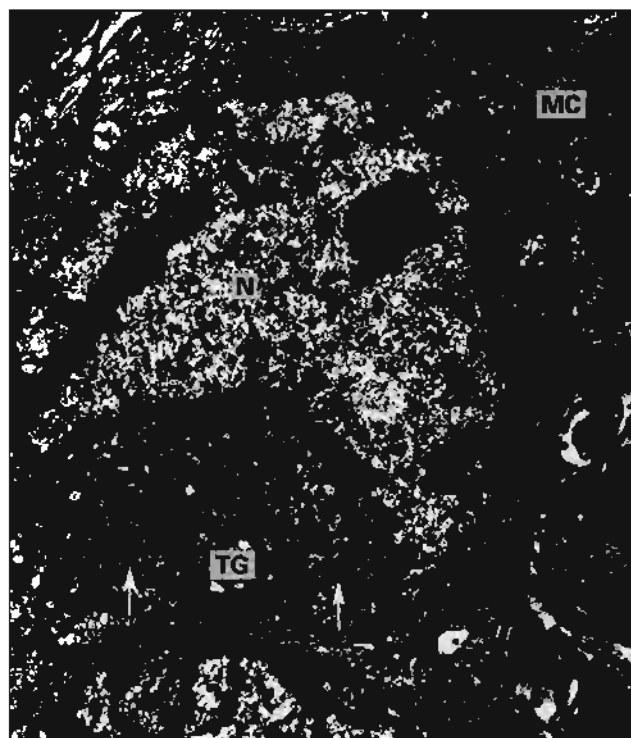


Fig 10-6b Characteristic features of Merkel cells (MC). (arrows) Large cluster of transmitter granules (TG). (N) Nucleus. (Original magnification $\times 12,600$.)

of neurotransmitters from Merkel cells. Destruction of Merkel cells abolishes slowly adapting (SA-I) responses in rats, suggesting that the Merkel cell is capable of mechanoelectrical transduction.³⁰

In humans and primates, Merkel cells are highly concentrated in the skin of the fingertips. Neurophysiologic studies of fingertip Merkel cell-nerve complexes have led to the conclusion that they provide tactile information for shape and texture perception.^{31,32} Linear responses over a wide range of indentations of the skin provide the brain with representation of the shape and surface characteristics of the object stimulus. Because of their relatively small receptive fields, the SA-I nerve endings also provide for two-point discrimination. It is proposed that the Merkel cell-nerve complexes of the oral mucosa play a similar tactile sensory function in recognition of particle size and texture during mastication of food.

Organized glial cell-encapsulated receptors

These receptors are most commonly located in the upper layers of the subepithelial connective tissue,

usually toward the crest of the dermal papillae.^{25,26,33} They are prominent in the lip, anterior palate, gingiva, and fungiform papillae of the dorsum of the tongue.³³⁻³⁵ Using the light microscope to visualize silver-stained tissues, histologists have described these structures as rosette-like, whirl-like, or ball-like nerve terminations. The nerve supplying the receptor is usually myelinated up to the point of entry into the glial cell-encapsulated end-organ.

Electron microscopy has shown that the basic encapsulated receptor consists of several nerve endings arising from branches of a single central axial nerve. The nerve endings are bulbous, contain many mitochondria, and are surrounded by glial cell cytoplasmic lamellae (Figs 10-7a and 10-7b).^{33,35} The capsular lamellae are cytoplasmic extensions of peripheral glial cells believed to be of Schwann cell origin.

There is considerable variation in the number and arrangement of the nerve endings and associated lamellae. The glial cell lamellae, arranged bilaterally around the nerve endings, contain numerous micropinocytotic invaginations (caveolae), cytoplasmic vesi-

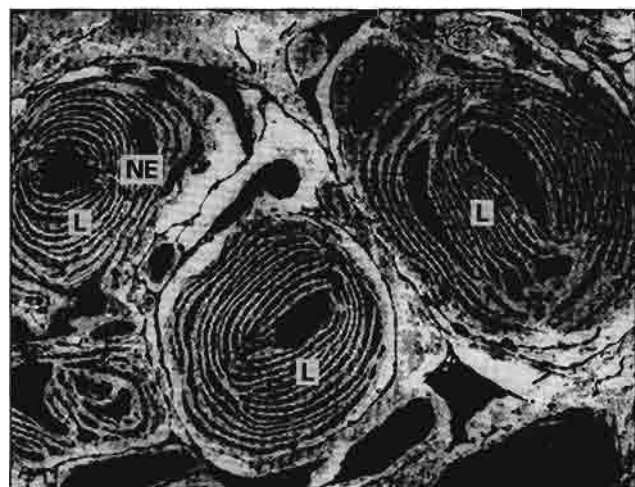


Fig 10-7a Electron micrograph of an encapsulated receptor. Encapsulated nerve endings (NE) characterize rapidly adapting mechanoreceptors in subepithelial connective tissue. (L) Lamellae. (Original magnification $\times 2,500$. Reprinted from Halata and Baumann³³ with permission from Springer-Verlag.)

cles, cytoplasmic filaments, and microtubules.³⁵ The extracellular spaces between the adjacent lamellae, as well as between the lamellae and the nerve endings, contain basal lamina material and fine collagen fibrils.³⁵

Cytochrome oxidase and acetyl cholinesterase activity have been localized in the nerve endings. The caveolae of the lamellar cytoplasm contain high levels of adenosine triphosphatase (potentially energizing a Ca^{++} pump). Calcium-binding proteins localized in the lamellar cells may participate in regulating the calcium concentration in the microenvironment of the nerve endings.

The strongest excitation of the receptor occurs during the moving (deformation) phase of the mechanical stimulus. The deformed state is normally followed by no response or by a rapidly adapting discharge. Thus, the organized encapsulated nerve ending is believed to be a rapidly adapting mechanoreceptor. This type of receptor is insensitive to static force. Encapsulation of the nerve ending is believed to impart a viscous adjustment property that minimizes responses to slowly applied mechanical stimuli and maximizes the effect of a rapidly applied force. This property also facilitates rapid recovery of the receptor.³⁶

Studies of rapidly adapting mechanoreceptors in insects indicate that the property of rapid adaptation

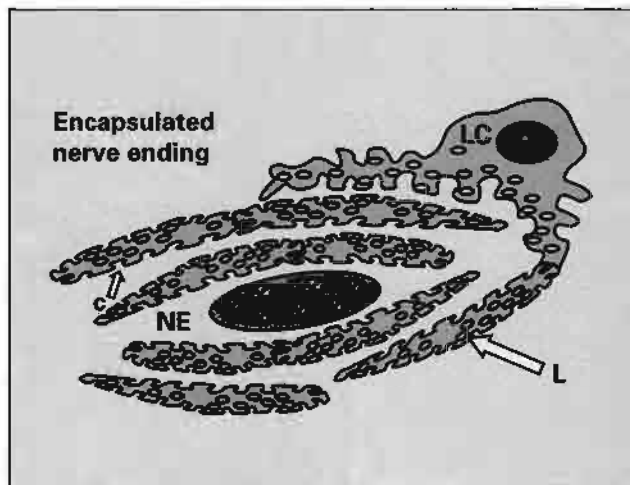


Fig 10-7b Structure of an encapsulated nerve ending. Thin cytoplasmic lamellae (L) arising from lamellar cells (LC) partially envelop the nerve endings (NE). The surfaces of the lamellae are studded with caveolae (c).

is the result of two components, an electrochemical component and a mechanical component. Direct electrical stimulation of the receptor neuron (bypassing any effect of the capsular structure) produced frequency responses similar to those elicited by normal mechanical stimulation. Rapid adaptation of mechanosensory neurons to electrical stimulation is caused by rapid inactivation, and slower reactivation, of voltage-gated Na^+ channels.³⁷

Meissner's corpuscles are widely distributed in skin, especially concentrated in the fingertips. They have been shown to be responsible for the detection of low-frequency vibrations and skin motion.^{2,31} It is thought that information from rapidly adapting afferents in the fingertip lead to feedback control of muscle activity needed for grip control and the delicate manipulation of handheld objects.² The Meissner's corpuscles of the oral cavity are most likely to have a feedback role in regulating the muscles of mastication, as well as in muscle function related to speech.

Ruffini-type mechanoreceptors

The Ruffini-type mechanoreceptor (SA-II) is designed to monitor tensional forces placed on collagen fiber bundles in connective tissues.² The parent axon of the Ruffini end-organ runs within nerve bundles that lie parallel to the long axis of major collagen bundles. Near its termination, the axon loses its



Fig 10-8a Ruffini-type receptors, characterized by an arborizing unmyelinated nerve that leads to a cluster of nerve endings (arrowheads) in close apposition to bundles of collagen fibers. A thin fibroblast capsule (arrows) partially demarcates the collagen fiber-nerve ending domain. (Original magnification $\times 3,200$. Reprinted from Halata and Baumann³³ with permission from Springer-Verlag.)

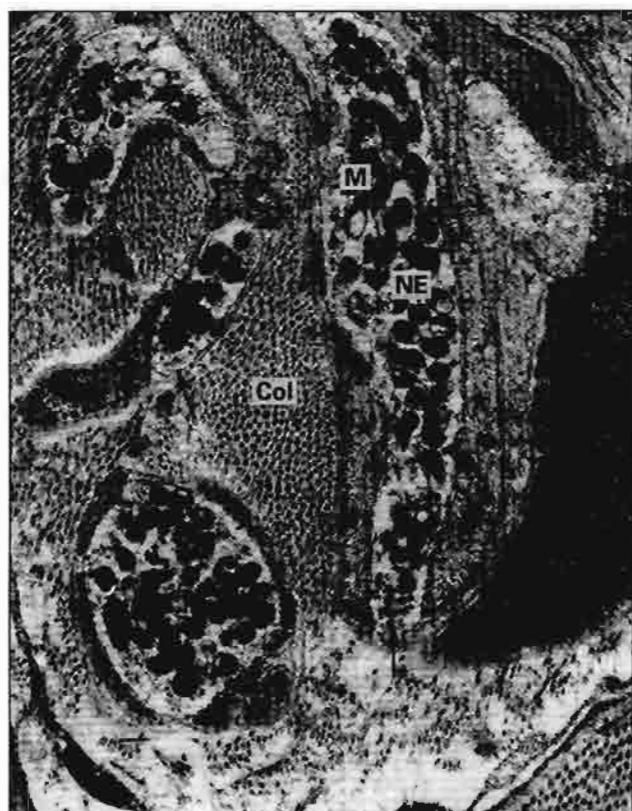


Fig 10-8b Nerve ending of the Ruffini-type receptor. Nerve endings (NE) contain many mitochondria (M) and are lined by cytoplasmic sheets (stars) from associated Schwann cells except for small zones of direct contact with extracellular space (arrows). (Col) Collagen fibers. (Original magnification $\times 14,000$. Reprinted from Halata and Baumann³³ with permission from Springer-Verlag.)

myelin sheath and branches several times to give rise to a group of nerve endings that are mostly aligned parallel to and in close contact with collagen fibers (Fig 10-8a).³³ Peripheral glial cell cytoplasm covers the terminal branches of the axon, except at the nerve endings, where small patches of the receptor are covered only by basal lamina material (Fig 10-8b).

The basal lamina covering of the nerve endings and small glial cell processes are apparently attached to adjacent collagen fibrils. When tensional loads increase in the direction of the long axis of the collagen fibers, the nerve endings and their associated glial cell coverings are stretched. Presumably, mechanical distortion of the neuronal plasma membrane alters the conformation and permeability of stretch-sensitive ion channels, leading to nerve depolarization and impulse generation. Of potential sig-

nificance to the function of mechanoreceptors of the Ruffini type is the recent finding that type IV collagen molecules regulate a stretch-gated sodium channel in *Caenorhabditis elegans*.³⁸ Deformation of the basal lamina could be transformed into a change in the patency of the channel protein.

In general, the overall shape of the Ruffini-type receptor conforms to the architecture of its surrounding collagenous tissue. In tendons, where the collagen fibers are aligned in close parallel bundles, the Ruffini mechanoreceptor is elongated so that its long axis is parallel to the major collagen fibers. In less organized tissues, the receptor nerve endings may have a treelike distribution. In skin and joint capsules, the Ruffini ending is surrounded by a thin, sleeve-like, connective tissue capsule.

The parent axon is of the A α or A β type. The cell bodies of oral Ruffini mechanoreceptors are present

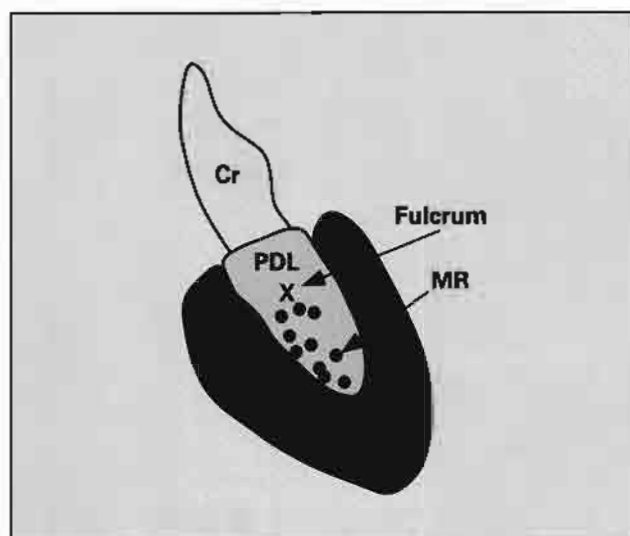


Fig 10-9 Location of Ruffini-type mechanoreceptors (MR) in the periodontal ligament (PDL) of the canine tooth of the cat. In general, the receptors are positioned apical to the fulcrum and within the mesiolabial quadrant of the ligament. Forces placed on the distolingual quadrant of the crown (Cr) elicit the greatest response. (AB) Alveolar bone. (Adapted from Linden et al.⁴¹)

in the trigeminal and mesencephalic nuclei of the fifth cranial nerve. Activation of the receptor, by stretching of the associated connective tissue, gives rise to an SA-II-type response, characterized by a slowly adapting, rather regularly spaced, impulse train.

Integration of inputs from SA-II endings in the temporomandibular joint and periosteal tissues, as well as in the periodontal ligaments, provide information on jaw position and force application during mastication and swallowing.

Periodontal mechanoreceptor nerve endings

The tactile response of a tooth is provided primarily by slowly adapting (SA-II) mechanoreceptors of the Ruffini type, located in the PDL.^{39,40} Anatomic and neurophysiologic studies have demonstrated a considerable variation among species and tooth types in the number, location, and response characteristics of periodontal mechanoreceptors. In the cat, an experimental animal frequently used for neurophysiologic studies, most periodontal mechanoreceptors are located in the canine tooth, especially in the area between the midregion (fulcrum) and the apical end of the PDL (Fig 10-9).^{40,41} Furthermore, the receptors tend to be preferentially placed along the mesiolabial side of the tooth. Physiologic studies in humans pro-

vide data that appears to support a similar distribution of receptors along the length and circumference of the root.

Periodontal mechanoreceptors exhibit directional sensitivity (Fig 10-10). Forces directed at the distolabial aspect of the crown of a tooth elicit the strongest response.⁴² This fact is in agreement with stretch-type mechanoreceptors concentrated in the mesiolabial PDL, below the fulcrum (see Fig 10-9). Periodontal ligament mechanoreceptors give a maximum response to stimuli applied to the tooth that contains them. These same receptors respond more weakly to forces applied to neighboring teeth (see Fig 10-10).

These multiple-tooth receptive fields are the result of mechanical linkage of adjacent teeth through coronal contact points and transseptal fiber bundles.^{43,44} For example, when force is applied to a lateral incisor, receptors in its PDL will be maximally activated, while receptors in the central incisor will be weakly activated because of contact (push) from the lateral incisor. Similarly, receptors in the canine tooth might also respond because of a pulling force generated through the transseptal fibers connecting it to the lateral incisor. Multitooth receptive fields exhibited by mechanosensitive neurons result from mechanical linkage of teeth and rarely result from branching of the terminal axons to supply several teeth.

The activation of the Ruffini nerve endings by slowly applied pressure (such as pushing against or pulling on a tooth) leads to reflex excitation of the jaw-closing muscles (Figs 10-10 and 10-11).⁴⁵ However, when force is applied at a rapid rate (for example, as a tap) to the crown of a tooth, an inhibitory reflex pathway to the jaw-closing muscles is activated. The short period of inhibition is followed by a longer, gradual excitation of the masseter muscle.

The receptors responsible for this response are believed to belong to rapidly adapting mechanoreceptors. Simple encapsulated receptors similar to the viscous adjustment-type mechanoreceptors of Meissner's corpuscles have been described in the PDL of some species, but not in humans. Because there is little anatomic evidence for encapsulated RA-II receptors in the PDL, it has been suggested that intradental A β nerve endings within the dentinal tubules might be responsible for the rapidly adapting receptor activity (discussed later in the chapter). Some neurons in the trigeminal ganglion demonstrate a rapidly adapting response to tooth tap stimuli, with characteristics unlike neurons of Ruffini receptors. However, the exact location of their receptor end-organs is still problematic.

Fig 10-10 (A) Response to a push-type stimulus of a single afferent nerve fiber of the inferior alveolar nerve applied to a mandibular central incisor and lateral incisor in each of four horizontal stimulation directions: (Li) lingual; (Me) mesial; (La) labial; and (Di) distal. Force is applied to plastic cubes cemented to the occlusal surface (teeth are viewed from above). Note the discharge pattern typical of a slowly adapting mechanoreceptor. The nerve fiber responds maximally when the lateral incisor is stimulated, indicating that the receptor is probably in the periodontal ligament of the lateral incisor. Force applied to the central incisor also stimulates the receptor. It is suggested that force is transmitted to the lateral incisor periodontal ligament receptor through the interdental transseptal fibers. (B) The greatest response (vectors) is to force applied from a distomesial direction. (c1) Contralateral central incisor; (MST) most sensitive tooth. (Reprinted from Trulsson⁴² with permission.)

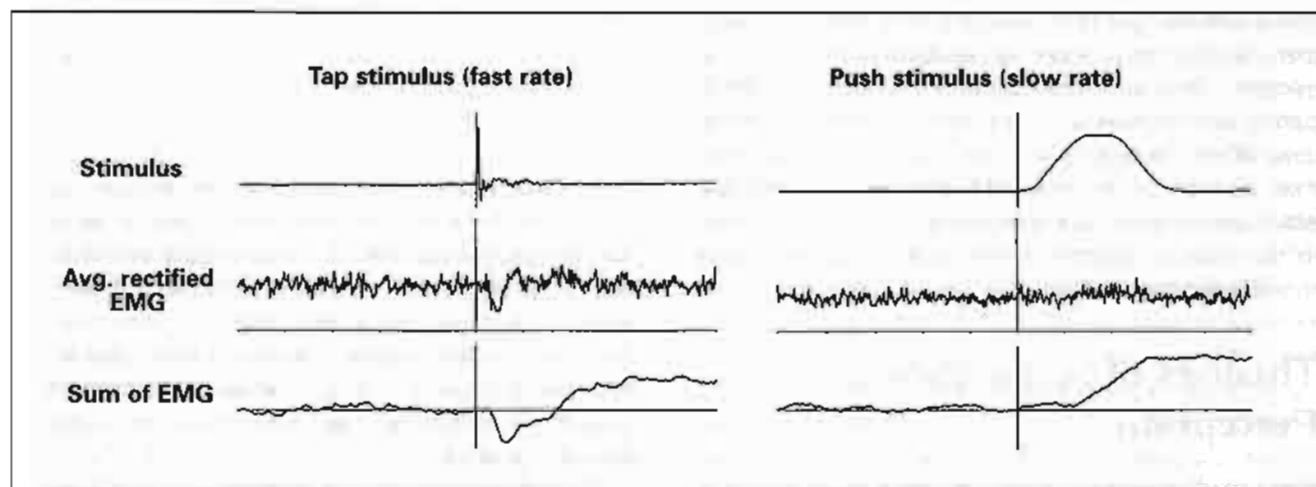
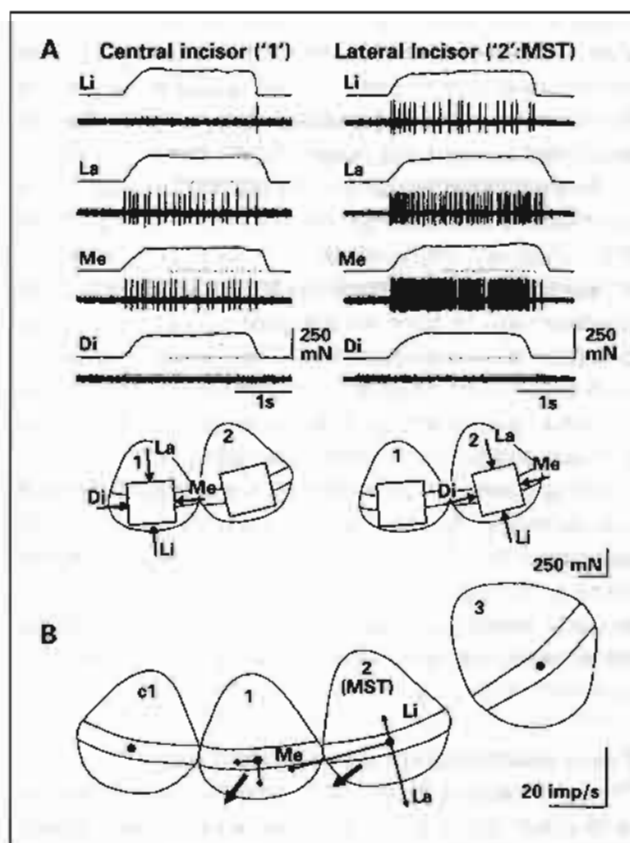


Fig 10-11 Reflex responses of human masseter muscle to mechanical stimulation of a tooth. The middle trace is the average (Avg) of the fullwave rectified surface electromyography (EMG), and the bottom trace is the cumulative sum of the EMGs. (Reprinted from Türker et al⁴⁵ with permission from Springer-Verlag.)

It has been suggested that fast-adapting responses might be attributed to differences in adaptation and response rates of PDL Ruffini-type mechanoreceptors based on their positions in the PDL relative to the fulcrum of the root. Receptors positioned closer to the fulcrum are expected to have a

faster adaptation and response rate than are receptors located near the apex.

The slow-rate receptors and the fast-rate receptors work together during chewing to permit holding and crushing of food while protecting the teeth from excessive rapid pressure applied by biting on rigid

objects. The slow-rate mechanoreceptors send input that facilitates the action of the jaw-closing muscles to hold and crush food, and the inhibitory actions of the fast-rate receptors prevent damage from the unexpected contact with a very hard object.

The replacement of natural teeth by implants and or dental prostheses decreases the level of oral tactile sensitivity. Implants are reported to be approximately 50 times less sensitive than natural teeth, and overdentures require even higher occlusal loading to produce a tactile response. The mechanosensitive end-organs responsible for tactile sensation induced by loading implants and dentures are located in periosteal and gingival connective tissues.

When teeth are extracted, PDL mechanoreceptors are destroyed but the neuronal cell bodies and their axons survive. The remaining axons supply the connective tissue and bone that fills the extraction socket. Whether new functional mechanosensitive nerve endings ever regenerate is an unanswered question at this time.

Encapsulated receptors of facial bones

These receptors have been observed histologically with silver stains in the periosteum and periarticular tissues of the facial bones. Morphologically, they resemble the encapsulated receptor illustrated in Figs 10-7a and 10-7b. Physiologically, they respond much like pacinian corpuscles as pressure and tension receptors. They are fast-adapting end-organs participating in the kinesthetic sensations of the facial muscles. When deformed, as during muscle contraction, they give an on-response. These receptors and the associated nerve networks are mostly concentrated in the medial regions of the frontal, maxillary, and mandibular bones.

Theories of Somatosensory Perception

Early studies of the peripheral anatomic units that form the basis of sensory perception led to the concept of specificity; ie, a single type of end-organ was responsible for a single sensation.²⁶ For example, free nerve endings were thought to be solely responsible for recording noxious stimuli, and encapsulated receptors were believed to be specific for mechanical stimuli. A specific end-organ was thought to be capable of only one form of response. This simplistic notion has been replaced by more complex hypotheses involving multiple modalities of response by end-organs as well as central summa-

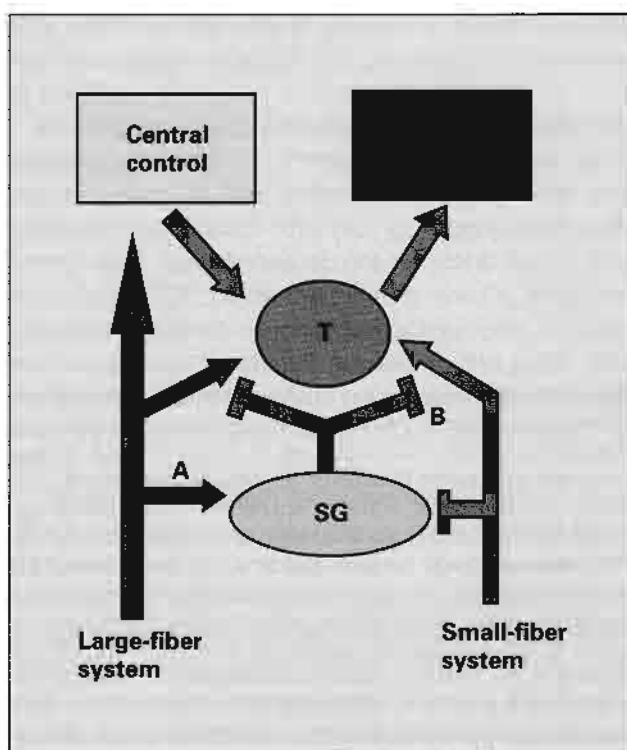


Fig 10-12 Basic aspects of the gate-control theory. Incoming signals on large-fiber somatosensory fibers have a stimulatory effect (A) on spinal gray (SG) interneurons, thereby acting pre-synaptically (B) to inhibit small-fiber activation of transmitting neurons (T) that conduct to higher action centers. Descending pathways from central control centers are also involved in an inhibitory modulation of the spinal somatosensory transmission neurons.

tion, integration, and modulation of afferent inputs from peripheral end-organs. In the central summation theory, pain is viewed not as a separate modality but rather as a result of overstimulation of other primary sensations. Receptors that are normally responsive to non-noxious stimuli can elicit pain when stimulated above a critical level or when pathologic conditions decrease their thresholds or enhance central summation of their input.⁴⁶

The gate-control theory of pain (Fig 10-12) arose from the discovery that rapidly conducting large-fiber ascending pathways inhibit activity in small-fiber pathways that primarily conduct noxious stimuli and the observation that descending central pathways in the brain stem also were capable of suppressing pain conduction.⁴⁷ According to the gate-control concept, afferent noxious impulses arriving at spinal or brain stem neurons are modulated, or "gated," by either facilitatory or inhibitory activity of other neurons prior to transmission to higher centers. The activity of large-diameter A β fibers that carry impulses gener-

ated by low-threshold non-noxious stimuli has an inhibitory effect via secondary neurons on small-fiber transmission of pain sensation to higher centers. On the other hand, impulses arriving from small A δ and C fibers that have been activated by intense noxious stimuli have a stimulatory effect (see Fig 10-12). Descending pathways in the periaqueductal gray matter also have a modulating effect in reducing transmission of noxious impulses from the brain stem nuclei.⁴⁸ Ren and Dubner⁴⁹ have reviewed the complexity of the modern concepts of descending inhibitory and facilitatory neural pathways for modulating peripheral nociceptor input.

Although the gate-control theory is an oversimplification of the complex interactions occurring between primary and secondary sensory neurons in the brain stem, it provides some explanation for the pain-modulating effect of acupuncture and transcutaneous electrical nerve stimulation.

Innervation and Sensation of the Pulp and Dentin

Johnsen⁵⁰ authored a well-illustrated review of the pattern of nerve distribution to the teeth. In the same issue of the *Journal of Dental Research*, Narhi⁵¹ reviewed the pre-1985 literature on the physiologic properties of the dental nerve fibers, and Olgart⁵² discussed the role of local factors in activating dental pain.

The nerve supply to the tooth consists of sensory axons from neurons in the trigeminal ganglion and autonomic fibers from neurons located in the superior cervical ganglion. Nerve bundles enter the tooth through the apical foramen and course axially toward the crown with little evidence of branching. On entering the coronal pulp, the nerves undergo repeated branching to give rise to a rich network of unmyelinated fibers within the subodontoblastic layer and the predentin (Fig 10-13).^{50,53-55} Nerve endings also penetrate the proximal portion of dentinal tubules.⁵⁰ Up to 100 dentinal tubules may receive nerve endings from a single afferent sensory nerve. The sensory nature of these fibers in mandibular teeth was confirmed by transection of the inferior alveolar nerve.⁵⁶ Anatomic studies and electrical recordings indicate that A β , A δ , and C fibers supply the teeth. The level of innervation is greatest in the pulp horns, where every other dentinal tubule appears to be innervated.^{50,54} Pulp cells have been shown to express messenger RNAs for neurotrophic factors and to stimulate neurite outgrowth from trigeminal neurons.⁵⁷

Recent studies have eliminated any doubt about the richness of this nerve network. The most convincing evidence that nerve endings of afferent fibers are present in dentin and predentin comes from tracer experiments.⁵⁸ Tritiated proline, microinjected into the brain stem nuclei of the trigeminal nerve, is taken up by neurons and subsequently transported antidromically to nerve endings in the pulp and dentin.^{53,58} Labeled nerve endings are subsequently identified by light and electron microscopic autoradiography. This method has revealed many labeled nerve endings in the predentin, the proximal end of the dentinal tubules, and the odontoblastic layer.

Additionally, immunocytochemical studies employing antibodies to neuroproteins have helped to characterize pulpal and dentinal nerve endings. Large-diameter myelinated sensory fibers, identified by immunoreactivity for parvalbumin in neuronal cell bodies and peripheral terminals, give rise to branching nerve terminals in the odontoblastic layer (Figs 10-13 and 10-14).

Numerous sensory nerves containing substance P and calcitonin gene-related peptide make up part of the subodontoblastic and odontoblastic plexus.⁵⁵ These nerve fibers begin migrating into the dental pulp at the completion of the bell stage of tooth formation.⁵⁵ As the crown nears completion, SP- and CGRP-positive fibers course toward the subodontoblastic and odontoblastic layers. Thin branching fibers join the subodontoblastic plexus and narrow fibers continue between the odontoblasts to terminate in predentin and the proximal parts of dentinal tubules.⁵⁸ Numerous beadlike nerve endings containing SP and CGRP appear to end in close apposition to the odontoblasts (see Fig 10-13). The CGRP-positive fibers outnumber those that contain SP.

Electron microscopic studies of dentinal innervation indicate that some unmyelinated nerves traverse the odontoblastic layer, coursing along the odontoblastic process in the proximal part of the dentinal tubules.⁵⁰ Many intratubular nerve endings contain small vesicles, some of which are of the dense-core variety, indicative of neuropeptide content.⁵⁰ Despite speculation that the odontoblast might act as a sensory transducer, there is no evidence of chemical synapses between nerve endings and odontoblasts. Although gap junctions are present in rather high numbers between odontoblasts and adjacent axon-like cytoplasmic processes, there is no good evidence that these cell processes are indeed small, unmyelinated nerves. Thus there is no convincing proof that electrotonic synapses (gap junctions) are formed between nerves and odontoblasts.⁵³

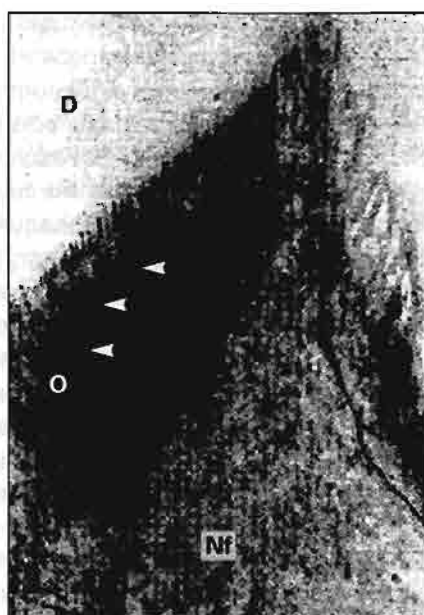


Fig 10-13 Substance P-immunoreactive nerve fibers (NF) and nerve endings (arrowheads) of the odontoblastic plexus (O) beneath a pulp horn. (D) Dentin. (Original magnification $\times 130$. Reprinted from Fristad et al⁵⁵ with permission from Elsevier Science.)

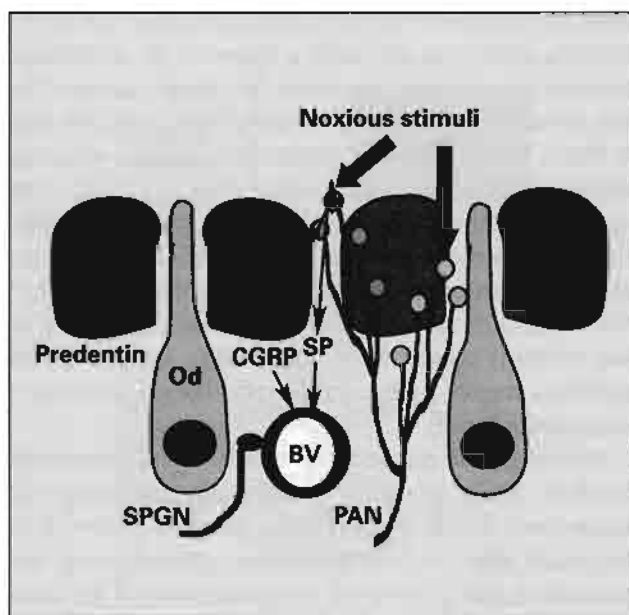


Fig 10-14 General position of afferent nerve endings in the odontoblastic layer (Od), the predentin, and the dentin. Noxious stimuli may activate the nociceptors via hydraulic movement of fluid in the dentinal tubules or by direct contact with nerve endings. Release of the neuropeptides, substance P (SP), and/or calcitonin gene-related peptide (CGRP) can produce vasodilation of local blood vessels (BV) and increase outward flow of fluid. (PAN) Primary afferent nociceptor; (SPGN) sympathetic postganglionic nerves.

No nerves have ever been reported in the outer parts of the dentinal tubules. The distal parts of the tubules are filled with a viscous fluid that may contain unmineralized collagenous matrix.

In the dental pulp, C fibers greatly outnumber A β and A δ fibers. The subodontoblastic plexus, including nerves that enter predentin and dentinal tubules, is made up of mostly the larger A β and A δ fibers, while most C fibers supply the pulp. The A δ - and C-fiber types relay pain back to central neurons in the trigeminal ganglion.

Recent physiologic evidence suggests that some dentinal nerve endings respond like rapidly adapting mechanoreceptors.⁵⁹ These responses are recorded from fast-conducting large, myelinated A β parent axons following activation by rapid mechanical transients (light taps) applied to the intact tooth. Steady force or displacement after initial contact does not cause further discharge. Unlike the mechanoreceptors of the PDL, these receptors are activated when force is applied in any direction. It is speculated that

axially aligned A β nerves in dentinal tubules are the anatomic units responsible for this rapidly adapting mechanoreceptive activity (Fig 10-15).⁵⁹ It has been suggested that the rigid walls of the dentinal tubules might be good transmitters of the mechanical energy needed to displace the nerve endings. Not all dental neurophysiologists accept the idea that the dentinal tubules house rapidly adapting mechanoreceptor nerve endings.

A substantial number of peripheral nerves supplying the dental pulp are autonomic fibers associated with blood vessels (sympathetic postganglionic nerves [SPGNs]; see Fig 10-14).⁶⁰ Physiologists have demonstrated a relationship between the sensitivity of pulpal nociceptors and intrapulpal pressure. Increases in blood flow and intrapulpal pressure intensify pulpal pain. In contrast, increases in sympathetic nerve stimulation, leading to decreases in pulpal blood flow, can diminish the response to chemical and thermal stimuli.

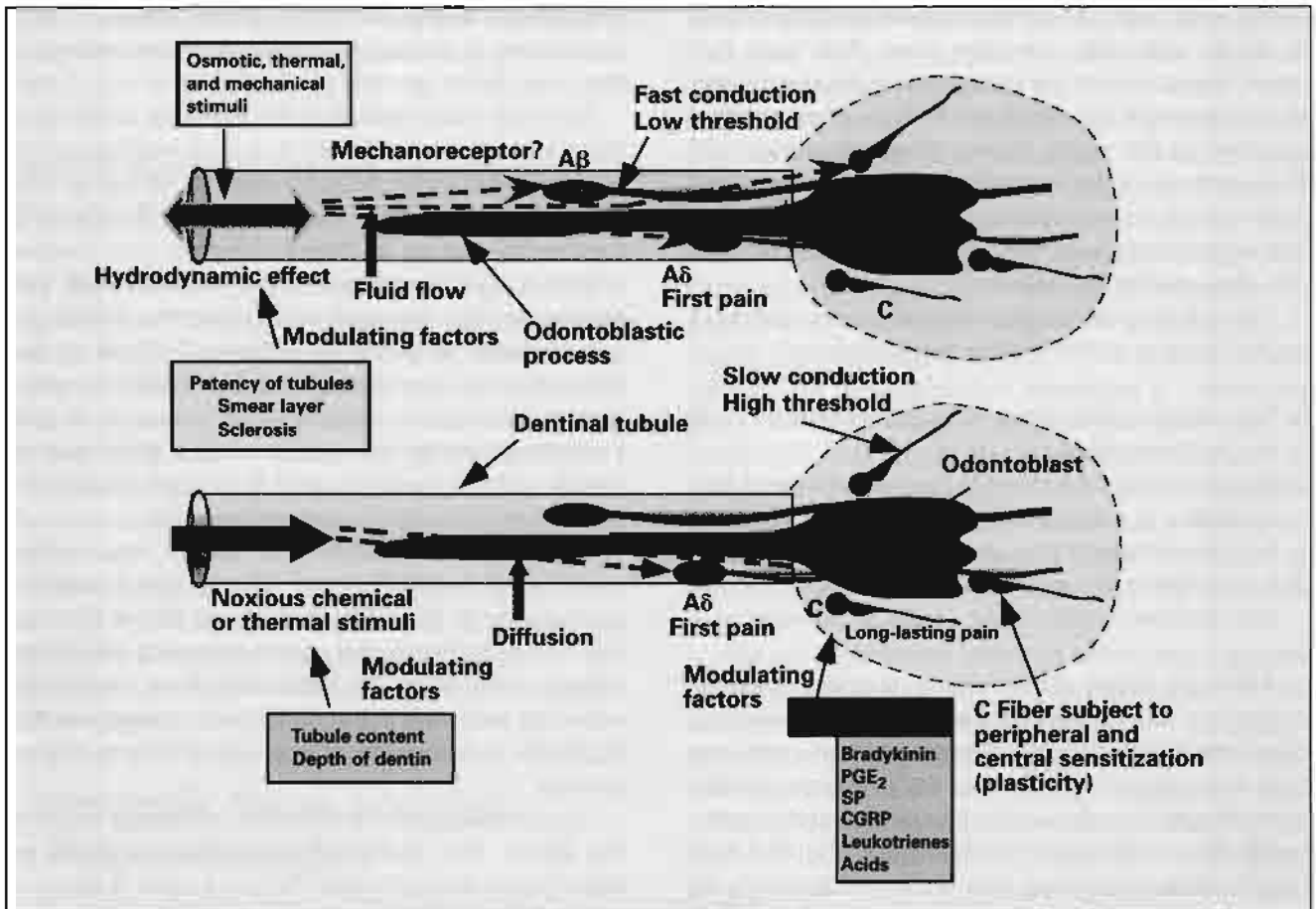


Fig 10-15 Current concepts of the generation of dentinal pain. Hydrodynamic distortion, thermal, and/or chemical stimulation of A δ - and C-fiber nerve endings in dentinal tubules and at the pulpodentin border cause pain. The degree of tubule permeability and the inflammatory state of the pulp are significant modulating factors. (CGRP) Calcitonin gene-related peptide; (FF) fluid flow; (NS) noxious stimuli; (PGE₂) prostaglandin E₂; (SP) substance P.

Dentinal pain

Generalized dental pain arises from the stimulation of nociceptors located within the tooth itself or within adjacent soft and hard tissues. In contrast, dentinal pain originates in the pulp, more specifically in dentin and the adjacent subodontoblastic nerve plexus. Dentinal pain input is transmitted along A δ -fiber mechano-thermal afferents and C-fiber polymodal afferents.^{61,62} Strong mechanical stimuli can elicit pain from high-threshold mechanoreceptive afferents.

Extensive experimentation has shown that the patency of dentinal tubules and the level of sensitivity of the dentinal nociceptor nerve endings influence the degree of pain perception. Dentinal pain is elicited through mechanical, chemical, and thermal disturbances of the dentin.⁶² In general, sharp pain is at-

tributed to stimulation of A δ nerve endings in the predentin and dentin, while longer lasting pain is believed to originate mostly from C fibers supplying the pulp proper (see Fig 10-15).⁶¹

Three hypotheses have been put forth to explain the mechanism of dentinal pain: the neural theory, the odontoblast transducer theory, and the hydrodynamic theory. According to the neural theory of dentinal pain, nerve endings located in the inner zone of dentin and in the predentin-odontoblast complex are directly activated by noxious chemical, mechanical, and thermal stimuli. This concept roughly parallels the condition known to exist in skin and oral mucosa, wherein A δ and C nociceptors respond to a variety of stimuli applied directly at a test site.

It is well known, however, that chemical, thermal, and mechanical stimuli applied to the surface of

dentin elicit pain. The dentinoenamel junction proves to be an especially sensitive zone. The facts that nerve fibers do not penetrate the outer dentin and that none reach the dentinoenamel junction create a problem for the neural theory. What can account for the sensitivity of the dentinoenamel junction and the outer dentin if nerve fibers do not reach that level? The odontoblast transducer and hydrodynamic theories may provide the answer.

The odontoblast transducer theory was advanced on the basis of the following observations:

1. The odontoblastic process occupies part or all of the dentinal tubule.
2. Gap junctions have been observed between odontoblasts and cytoplasmic processes interpreted to be unmyelinated nerve endings.
3. It is probable that odontoblasts are hyperpolarized and capable of generating electrical currents.

Although these observations suggest a transducer role for the odontoblasts, there has been no direct electrophysiologic recording of transducer activity from these cells, nor has the presence of electrotonic (gap) junctions between nerves and odontoblasts been confirmed. Furthermore, the fact that pulpal tissue containing necrotic odontoblasts is responsive to noxious stimuli decreases the significance of odontoblast transducer activity.

In contrast, the hydrodynamic theory has considerable experimental support.^{61,63} Osmotic pressures within the confines of the dentinal tubules generate fluid flow and mechanical disturbances in dentin and the adjacent predentin zone. Outward movement of dentinal fluid is known to be especially painful. Osmotically generated fluid flow could create local disturbances of the axonal plasma membrane and activate stretch-sensitive ion channels, leading to depolarization and the generation of action potentials. The rate of displacement of fluid in the tubules appears to be an important element in activating nerve endings. Clinical experience has shown that substances that lead to dehydration, evaporation, and/or thermal changes in the dentinal fluid act as noxious stimuli.

Although the hydrodynamic theory provides an attractive explanation for dentinal pain, it represents an oversimplification of a complex phenomenon, for it is known that not all substances that cause a change in osmotic pressure lead to dentinal pain.⁶⁴ Furthermore, some chemical agents that do not alter osmotic pressure are able to produce dentinal pain when applied to the surface of freshly cut dentin.

Presumably these chemicals diffuse through dentinal tubules to contact and activate nerve endings in the inner dentin and the predentin.

The best interpretation of the available evidence is that dentinal pain is elicited by a dual mechanism involving mechanical displacements of A δ nerve endings by osmotic fluid movements in the dentinal tubules as well as by direct chemical and thermal effects on C-fiber nerve endings. It is also likely that severe osmotic changes may distort the peripheral pulpal tissue to indirectly activate C fibers of the pulp. Dentinal or pulpal pain is modulated by intrapulpal pressure. Increased blood pressure and/or increased thermal stimulation of the pulp lead to higher pulpal pressure, which in turn causes increased neural activity and dentinal pain.

Impulses from dental and pulpal nociceptors travel on A δ and C fibers to the trigeminal ganglion and beyond to the brain stem nuclei of the fifth cranial nerve. Gating activity from incoming peripheral stimuli along large A β fibers and from central descending pathways in the periaqueductal gray matter suppress transmission of noxious activity to higher centers.

Dental nociception is increased following injury to the dentin. This state of hyperalgesia might be related to structural changes that are known to occur in the peripheral nerve supply during recovery from the injury. Within hours after injury to dentin, new nerve endings sprout from the axons supplying the odontoblastic and subodontoblastic cell layers localized to the zone of damage.⁶⁵ The new nerve endings are primarily CGRP positive.⁶⁵

A concomitant increase in the expression of NGF in the fibroblasts of the subodontoblastic layer suggests that the local release of NGF may trigger axonal sprouting. This hypothesis is supported by the fact that the new nerve endings contain high levels of NGF receptor. Of interest is the observation that steroid administration reduces the number of new nerve endings by 50%.

It has been suggested that the rapid increase in new nerve endings following injury might be responsible, in part, for dentinal hyperalgesia. A potential link between increased expression of NGF and hyperalgesia is suggested by the observation that the sensitivity of A δ nerve endings is increased several fold by NGF. Injury to peripheral nerve endings also leads to chemical changes in the centrally located neuronal cell bodies, mediated by anterograde transport of NGF from the peripheral nerve endings of inflamed tissues.⁶⁶⁻⁶⁸ These changes lead to alteration of information processing and increased nocicep-

tion. Additional discussion of nerve sensitization and hyperalgesia is presented in "Basic Science Correlations," later in this chapter.

Nerve Regeneration Following Tooth Extraction

The extraction of a tooth is similar to a nerve amputation injury. The nerve endings are severed but the cell bodies, located in the trigeminal nucleus and the mesencephalic nucleus, survive the injury and are able to mount a regeneration process. Peripheral nerves regenerate in the extraction socket within 1 month following injury. Regenerated nerves innervate the bone and connective tissue that replaces the lost dental unit.

Clinical reports indicate that abnormal nerve regeneration may produce neuromas in extraction sites and that in a very small percentage of patients these neuromas can cause subsequent idiopathic trigeminal neuralgias.

Peptidergic Nerve Endings in Pulp and Gingiva

Sensory nerve fibers that contain CGRP and SP participate in vasodilation and neurogenic inflammation.⁶⁴ The dental pulp, and especially the subodontoblastic plexus, is supplied by such fibers.^{58,69} The release of CGRP and SP during nerve injury may help to mediate the wound-healing response by stimulating the formation of new blood vessel and reparative dentin (see "Primary afferent nociceptor neuropeptide effector activity," later in this chapter). These neurotransmitters may also generate increased outward fluid flow in the adjacent dentinal tubules.

Basic Science Correlations

Biology of nociceptor sensitization

Increased excitability of primary afferent nociceptor (PAN) leads to the clinical condition of hyperalgesia, a common aftermath of pulpal inflammation. In the hyperalgesic state, dental nociceptors are triggered by relatively low-level stimuli. For example, a mildly hot but otherwise innocuous beverage can trigger severe pain in a tooth that has an inflamed pulp.

Sensitization develops when PANs have been excessively stimulated or when endogenous peptides,

acid, and/or other chemical substances generated in damaged and inflamed tissue interact with receptors and/or enzymes located in the PAN plasma membrane (Fig 10-16).^{70,71} Primary afferent nociceptors that have become sensitized exhibit increased spontaneous discharge, decreased activation threshold, and a prolonged response to a suprathreshold stimulus (Fig 10-17).^{46,72} Histamine, serotonin, bradykinin, prostaglandin E_2 (PGE_2), neutrophil chemotactic peptides, and interleukin 1 have the potential of acting as PAN-sensitizing agents.^{15,71} In sites of inflammation, the plasma protein kininogen is cleaved by the proteolytic enzyme kallikrein to give rise to bradykinin (see Fig 10-16). Binding of bradykinin to bradykinin receptors on the PAN plasma membrane activates signal transduction pathways involving increased production of diacylglycerol, activation of protein kinase C, and an increase in Na^+ conductance.⁷³

Various substances acting as chemotactic factors for neutrophils, such as the C5a component of the complement system, leukotriene B_4 , and the tripeptide *N*-formyl-methionyl-leucyl-phenylalanine, stimulate the production of (8R,15S)-dihydroxyeicosa-(5E-9,11,13Z)-tetraenoic acid (8R,15S-diHETE) by neutrophils.^{15,71} Both PGE_2 and 8R,15S-diHETE, products of arachidonic acid metabolism in activated neutrophils, are potent sensitizing agents that increase the concentration of the second messenger cyclic adenosine monophosphate (cAMP) in PANs through signal transduction pathways involving stimulatory G proteins and adenylyl cyclase (Figs 10-16 and 10-18).⁷⁴ Bradykinin and interleukin 1 have been shown to stimulate the production of PGE_2 in sympathetic postganglionic neurons. Prostaglandin E_2 released from SPGNs and cells of the inflammatory infiltrate is free to act as a sensitizing agent on adjacent PANs (see Fig 10-16).

Additional bioactive peptides are produced by cleavage of phospholipase A-activating protein by a convertase enzyme (Fig 10-19).⁷⁵ Phospholipase A-activating protein is found in many cell types, including T cells, monocytes, macrophages, endothelial cells, and muscle cells. Biologically active peptide fragments of phospholipase A-activating protein activate phospholipase A2, an enzyme that attacks cell membrane lipids, providing substrates for the generation of eicosanoid mediators of inflammation. Some peptides derived from phospholipase A-activating protein act as activators of stimulatory G proteins, while others are able to create temporary pores in the plasma membrane that permit ion exchange.

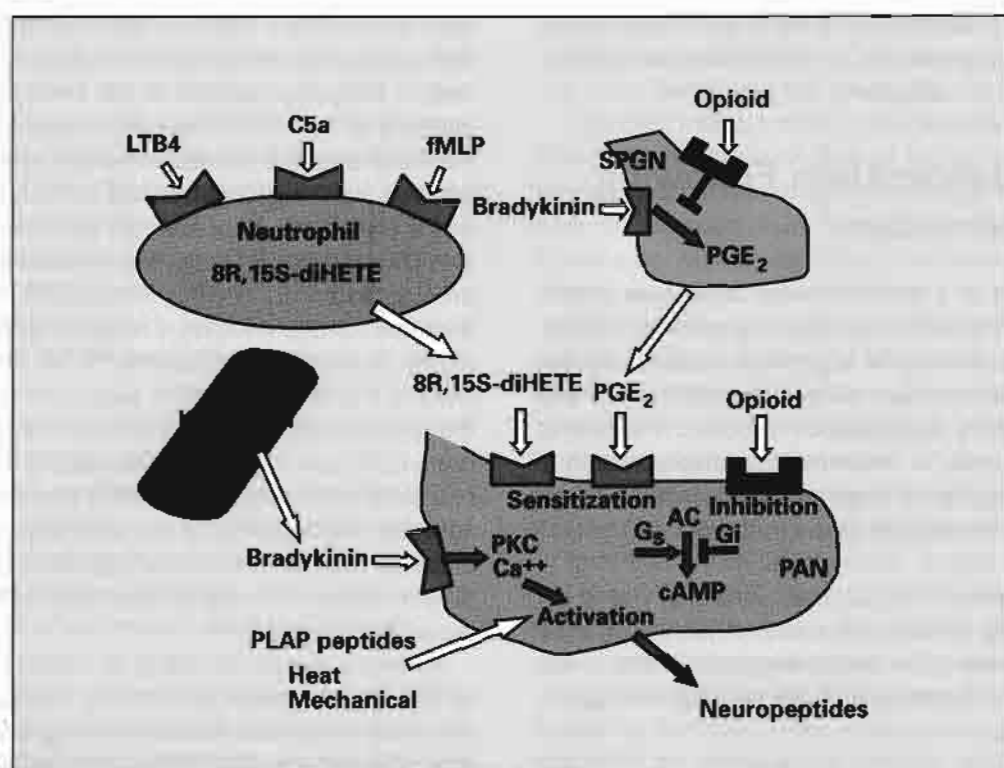


Fig 10-16 Potential mechanisms that lead to the sensitization of primary afferent nociceptors (PAN). Neutrophils responding to chemotactic factors, such as leukotriene B₄ (LTB₄), the C5a component of the complement system, and the tripeptide *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), generate fatty acid derivatives of arachidonic acid metabolism. Two of these products, (8R,15S)-dihydroxy-eicosa-(5E-9,11,13Z)-tetraenoic acid (8R,15S-dihETE) and prostaglandin E₂ (PGE₂), increase PAN sensitivity by interacting with receptors on the PAN plasma membrane that activate G proteins and increase cyclic adenosine monophosphate (cAMP). Bradykinin, a product of the proteolysis of plasma kininogen, also has a sensitizing action. It activates phosphokinase C (PKC) and increases the concentration of free calcium in the cytoplasm. Activated PANs respond by releasing a variety of neuropeptides that have significant vasodilator and proinflammatory effects. Opioids act to counter the activation of PANs by activating inhibitory G proteins. Phospholipase A-activating protein (PLAP) enters this cascade as shown in Fig 10-19. (AC) Adenyl cyclase; (BV) blood vessel; (Gi) inhibitory G protein; (G_s) stimulatory G protein; (SPGN) sympathetic postganglionic neuron. (Adapted from Levine et al⁷¹ with permission from the Society of Neuroscience.)

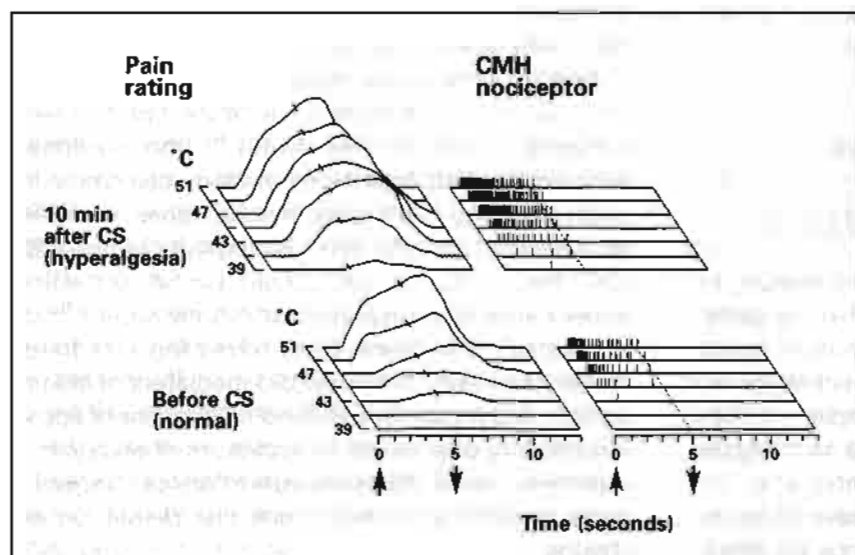


Fig 10-17 Pain rating to heat stimulation in human subjects. Results indicate an increasing magnitude of pain in hyperalgesia. Pain is increased and longer lasting (indicated by the height and length of curves) in the hyperalgesic state than in the normal state at similar stimulus temperatures. Right panel shows action potentials in the chemical, mechanical, and heat-sensitive (CMH) nociceptors. (CS) Conditioning stimulus. (Reprinted from LaMotte⁷² with permission.)

Fig 10-18 Sensitization of primary afferent nociceptors (PANs) via arachidonic acid metabolites. (LTB₄) Leukotriene B₄; (8R,15S-diHETE) (8R,15S)-dihydroxyeicos-5E-9,11,13Z-tetraenoic acid.

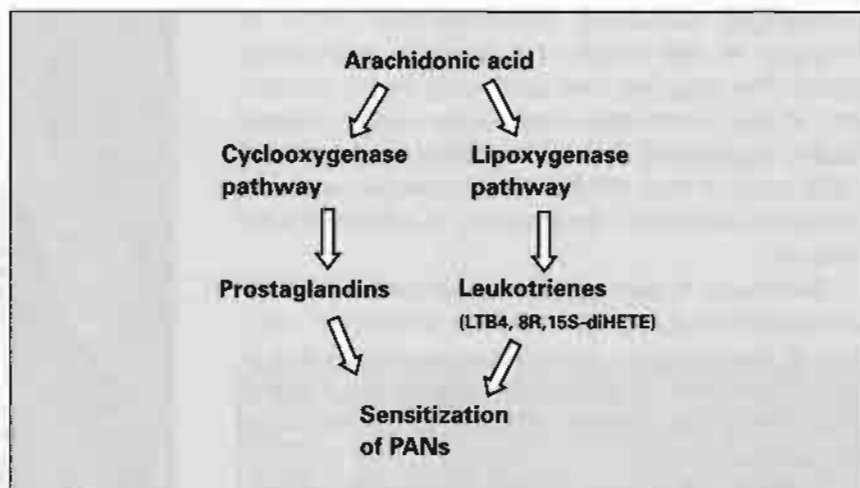
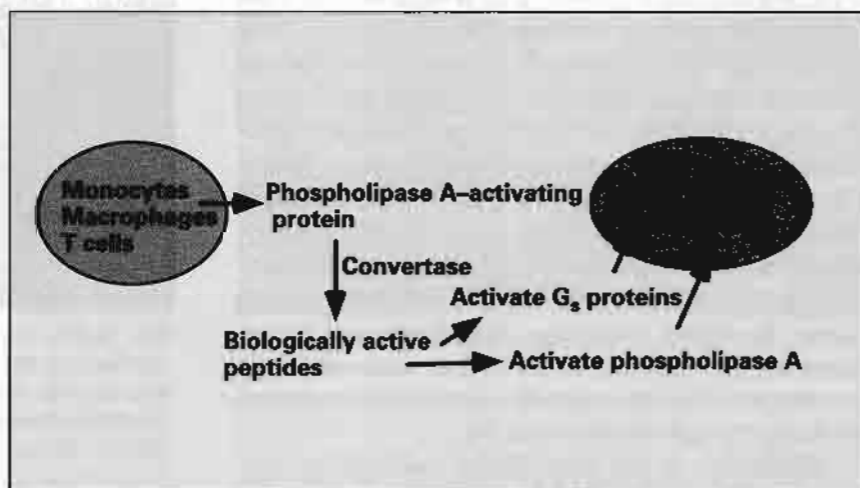


Fig 10-19 Nerve ending sensitization by the phospholipase A-activating protein (PLAP). Numerous cell types, including monocytes and macrophages, produce PLAP. This protein is broken down by a convertase enzyme into smaller biologically active peptides. These peptides have been shown to activate stimulatory G (G_s) proteins and to activate the phospholipase A enzyme that generates lipid metabolites for the eicosinoid synthetic pathways. Both of these cascades can lead to the activation or sensitization of nerve endings.



Indomethacin and other inhibitors of prostaglandin synthesis inhibit the hyperalgesia induced by interleukin 1 β . The anti-inflammatory action of aspirin also acts to inhibit the development of hyperalgesia.

Substances that have an antisensitizing effect also modulate the activities of PAN and SPGN terminals. Opioids interact with receptors in the PAN and SPGN plasma membranes to activate inhibitory G proteins that inhibit the production of cAMP. Prostaglandin synthesis is also decreased by opioids. Opioid-binding proteins are manufactured in the centrally located cell bodies and transported to the peripheral nerve terminals. The spontaneous activity of hyper-sensitized nociceptors is decreased by opioids.⁷⁶ Injection of exogenous opioids into inflamed joints has been used successfully to reduce pain.

Primary afferent nociceptor neuropeptide effector activity

In addition to their primary nociceptive functions, PAN nerve endings are also a neuroeffector organs, capable of releasing neuropeptides that influence the behavior of neighboring cells and blood vessels. Activated PANs release the neuropeptides SP and CGRP, which influence the behavior of adjacent cells.^{15,71,77} The release of SP after noxious stimulation or nerve damage produces widespread effects, leading to neurogenic inflammation. Substance P and CGRP cause vasodilation and increased vascular permeability.

The increased vascular permeability produced by SP is due to the stimulation of nitric oxide production in endothelial cells. Nitric oxide acts as a second

messenger, activating pathways that result in changes in cell shape and widened intercellular clefts. The enzymes that participate in the production of nitric oxide have been located within odontoblasts, suggesting that odontoblasts might release nitric oxide in their immediate environment and participate in altering the permeability of adjacent blood vessels.

Substance P also acts as a chemoattractant for neutrophils and it potentiates the phagocytic function of macrophages and neutrophils. Production of PGE_2 , interleukin 1, and tumor necrosis factor α and secretion of collagenase are elevated in white blood cells exposed to SP.

Pulpal blood vessels, including those in the odontoblastic plexus, undergo vasodilation in response to electrical stimulation of teeth and/or the afferent nerves leading from teeth. A similar response is produced by mechanical irritation of dentin. The vasodilation that follows stimulation of tooth sensory afferents is due to the release of CGRP and SP from C-fiber nociceptor nerve endings. Vasodilation of pulpal blood vessels increases intrapulpal hydrostatic pressure, which in turn causes the flow of dentinal fluid to increase. It has been suggested that the reflex rise of the outward flow of dentinal fluid is a protective response to the penetration of dentin by irritant molecules. Extravasation of serum proteins from dilated postcapillary venules within the odontoblastic plexus might also accelerate a protective occlusion of open dentinal tubules.

Modulation of the vasodilation response to neuropeptide release from PANs is the result of vasoconstriction, produced by the release of noradrenaline from SPGNs. The release of noradrenaline can occur as a result of antidromic stimulation of SPGNs induced via sensory afferent nerve reflexes or because of the direct activation of the SPGN nerve ending by bradykinin.

Clinical Correlations

Taste receptors

Mammalian taste buds are composed of specialized epithelial taste receptor cells. They are found primarily on the tongue and in smaller numbers in the epithelium of the glossopalatine arch, soft palate, posterior surface of the epiglottis, and the posterior wall of the pharynx. Large numbers of taste buds are located in the circumvallate, fungiform, and foliate papillae of the tongue. The total number of taste buds ranges from 4,000 to 6,000; the highest con-



Fig 10-20 Taste buds (TB) in the wall of the circumvallate papilla. Each taste bud is surrounded by keratinocytes (K) and extends from the surface of the epithelium to the underlying connective tissue (CT). (Toluidine blue stain. Original magnification $\times 800$.)

centration is found in the circumvallate papillae. Each taste bud contains 100 to 150 taste cells. Taste receptor cells express keratins 7, 8, 18, and 19, which are not detected in adjacent keratinocytes.⁷⁸⁻⁸⁰

Tastants are perceived as either sweet, sour, salty, umami (amino acid), or bitter. These five taste qualities are the perceptual and psychophysical results of the complex integration of inputs from taste receptor cells.⁸¹ The output of each cell is the summation of the activity of ion channels and receptor signaling pathways triggered by tastant molecules. Most taste fibers respond to several of the five taste modalities but demonstrate differentiation by developing a lower threshold for one type of stimulus.^{81,82} For example, a single taste nerve may show weak responses to sweet, salty, umami, and sour stimuli and a stronger response to bitter tastants.

Despite the fact that all regions of the tongue and all individual taste buds respond to chemicals in all five taste categories, different areas on the tongue



Fig 10-21a Electron micrograph of a longitudinal section of mouse fungiform taste bud demonstrating basal (B), dark (D), light (L), and transducer (type III) cells (G). The dark and light cells are thought to represent types I and II cells, whose function is still problematic. At the apical pole, all the cells (except for basal cells) terminate in microvilli that project into the taste pit. Keratinocytes (K) are aligned along the lateral borders of the taste bud. (Original magnification $\times 5,000$. Reprinted from Seta and Toyoshima⁸³ with permission from Springer-Verlag.)

have decreased thresholds for one or more of the five taste qualities. Regional patterns of taste sensitivity to the five taste qualities are apparent on the tongue of rats (an animal often used in taste experimentation) but less pronounced in humans.⁸¹

Each taste bud extends from the basement membrane to the surface of the oral epithelium. At the apex of the bud, there is an opening in the keratinocyte layer (the taste pore), through which the fluids of the oral cavity can communicate with the taste pit (Figs 10-20 and 10-21).⁸¹ Taste receptor cells extend cytoplasmic processes into the taste pit. At the base of the taste bud, numerous unmyelinated nerve fibers penetrate the basal lamina to come into close contact with the taste cells. The connective tissue be-

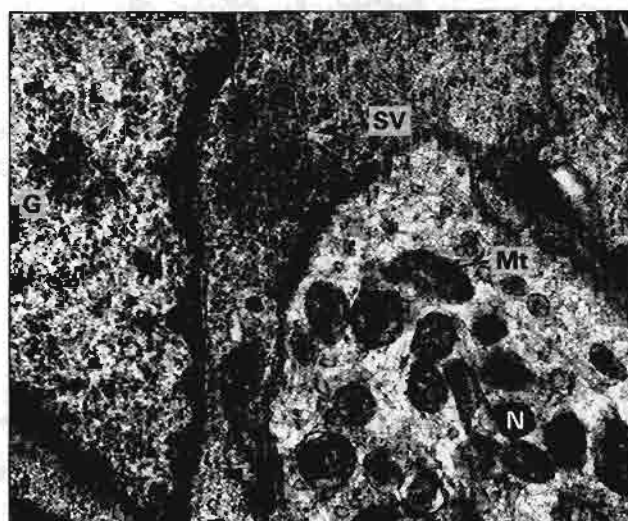


Fig 10-21b High magnification of a chemical synapse between a transducer cell (G) and a nerve (N) ending. High concentrations of small clear synaptic vesicles (SV) and dense-core granules are concentrated opposite the nerve terminal. (Mt) Mitochondria. (Original magnification $\times 5,000$. Reprinted from Seta and Toyoshima⁸³ with permission from Springer-Verlag.)

neath the taste buds is richly innervated and highly vascularized. Taste buds on the anterior two thirds of the tongue are innervated by branches of the seventh facial nerve, while taste buds located in the posterior part of the tongue and on the soft palate and epiglottis are innervated by the ninth and tenth cranial nerves.

For many years, taste buds were described as being composed of two cell types: supporting or sustentacular cells, appearing as darkly stained cells, and neuroepithelial or gustatory cells, appearing as larger and less intensely stained cells. The advent of electron microscopy led to a better understanding of taste bud structure. Most anatomists agree that taste buds contain four types of cells.

Type I cell

This cell reaches from the base of the taste bud to the taste pit, where it terminates into numerous microvilli that extend into the taste pit (see Figs 10-21a and 10-21b).⁸³ In routine histologic and electron microscopic preparations, it appears as a darker-staining cell type. The increased density of the cytoplasm is caused by the higher concentration of cytoplasmic filaments in this cell type. The apical cytoplasm contains many vesicles and dense granules. The cell contains a moderate amount of rough endoplasmic reticulum and a Golgi complex oriented toward the taste pit.

The function of this cell type is believed to be the synthesis and secretion (via the dense granules) of substances into the taste pit area. The taste pit contains an electron-dense, carbohydrate-rich substance that might act as an ion exchange resin in regulating the entry of certain molecules into the taste pit while excluding others. Some investigators have suggested that the content of the taste pit might have cleansing and/or antibacterial properties responsible for preventing bacterial colonization of the taste pore.⁸⁴

The apical part of the type I cell is joined to other taste cells by zonula occludens and zonula adherens. At its basal surface it is in close contact with unmyelinated nerve fibers. These fibers are presumed to be efferent fibers, because there are no neurosecretory transmitter vesicles in the adjacent cytoplasm of the type I cell.

Type II cell

The type II cell is larger and lighter staining. The apical cytoplasm gives rise to several microvilli that are shorter and broader than those of the type I cell. These microvilli project only to the base of the taste pit. There are no dense granules in the Golgi complex or in the apical cytoplasm.

Type III cell

Numerous small, dense-cored granules and clear vesicles, largely located in the basal cytoplasm, characterize these cells. The granules and vesicles are especially numerous adjacent to synapselike contacts between the type III cell and adjacent nerve endings (see Figs 10-21a and 10-21b).^{83,85} Although the type III cell was previously described as the only type to form synapses to afferent fibers, Gilbertson et al⁸¹ have stated that all taste bud cell types form synaptic junctions with afferent fibers. Thus all cell types may be considered to have receptor functions.

The apical end of the cell contains a single broad cytoplasmic process that extends into the taste pit beyond the dense material (see Fig 10-16). Changes in the permeability of ion channels and activation of signal transduction mechanisms triggered by tastant molecules occur at the plasma membrane of the cell process that extends into the taste pit. Studies of the dense-cored vesicles found at the base of the type III cell indicate that they contain a biogenic amine, probably 5-hydroxytryptamine (serotonin), that could act as a neurotransmitter substance.⁸⁶

Type IV cell

Type IV cells are smaller, less differentiated cells located along the base of the taste bud. Because they are preferentially labeled with tritiated thymidine, they are believed to represent a pool of stem cells that give rise to cell types I, II, and III.

Taste bud cell turnover occurs within 2 weeks. Taste cell differentiation is still a controversial subject. Some investigators believe that cell types I to III represent stages in receptor maturation rather than separate cell lines.

Taste transduction mechanisms

Ions and chemicals contained in food stimulate taste sensation by interacting with ion channels and metabotropic receptors concentrated in the plasma membrane of the apical cytoplasmic process of transducer cells. Disruption of ion traffic through specific plasma membrane channels leads to direct changes in cytosolic ion concentrations, while interaction at metabotropic receptors activates signal transduction pathways that secondarily alter ion concentrations in the cytoplasm. Shifts in cytoplasmic ion concentrations lead to plasma membrane depolarization, generation of action potentials, and the release of neurotransmitters at the chemical synapses with taste nerve fibers.

Two families of metabotropic receptors have been recently identified as candidates for transduction of sweet, bitter, and umami (amino acid) tastants.⁸⁷ These are the T1Rs and T2Rs in the superfamily of G protein-coupled receptors. The T1R2 and T1R3 members of the T1R group act as transducers of sweetness, while the T2R family reacts to bitter compounds. The T1R1 and T1R3 proteins have been proposed to function as amino acid receptors, responsible for the umami taste.⁸⁷

The five basic taste modalities generate sensory input through different signaling mechanisms (Fig 10-22). Sour taste is caused by acidic foods. The

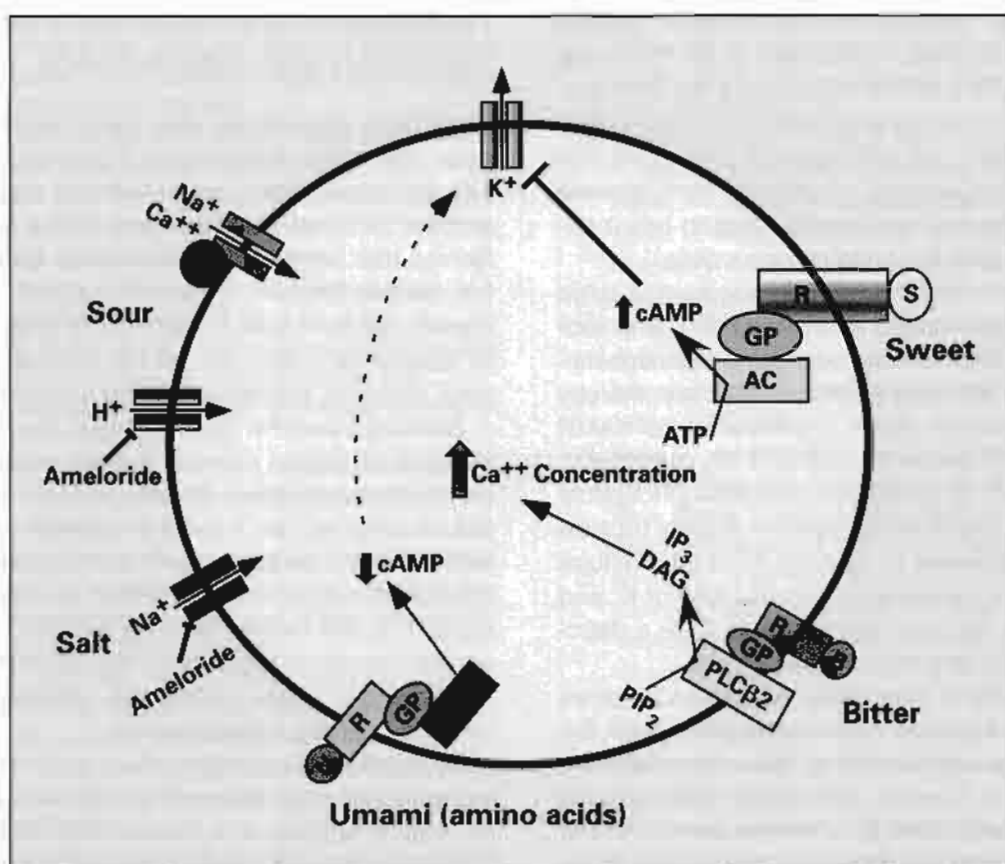


Fig 10-22 Plasma membrane transducer systems and the interacting taste modalities. Sour substances generate H⁺ that blocks pH-sensitive potassium channels and activates proton-gated cation channels. Salty substances lead to increased Na⁺ fluxes through amiloride-sensitive sodium channels as well as the activation of sodium-gated cation channels. Sweet substances trigger increased cytosolic cyclic adenosine monophosphate (cAMP) via activation of guanosine triphosphate-binding proteins (GP) and adenylyl cyclase (AC). Bitter chemicals increase diacylglycerol (DAG) and inositol triphosphate (IP₃) through activation of phospholipase Cβ2 (PLCβ2). The ultimate effect in each of these systems is the depolarization of the plasma membrane and the formation of action potentials. The diagram represents a summary of current thinking in a rapidly changing field—there is considerable variation and overlap among species. (ATP) Adenosine triphosphate; (PDE) phosphodiesterase; (PIP₂) phosphatidylinositol-4, 5-bisphosphate; (R) receptor.

H⁺ ions (protons) in the sour food penetrate the taste receptor cell through the epithelial amiloride-sensitive sodium channel. Protons may also activate H⁺-gated cation channels, leading to inward flow of Na⁺ and Ca²⁺ ions. Furthermore, protons may block outward flow of K⁺ ions through H⁺-gated K⁺ channels. These pathways tend to depolarize the taste receptor cells and release neurotransmitters.^{81,88}

A second mechanism proposed for detection of acidic stimuli involves a decrease in intracellular Ca²⁺ concentration activated via a G protein-coupled re-

ceptor.⁸⁹ This pathway is believed to be dependent on regulation of the release and/or sequestration of Ca²⁺ from intracellular storage sites.

Sodium ions responsible for salty taste stimuli move passively into the cell through amiloride-sensitive sodium channels. The inward flux of Na⁺ depolarizes the cell membrane, leading to release of neurotransmitters.

Sugars bind to metabotropic receptors (T1R2 and T1R3) linked to trimeric guanosine triphosphate-binding proteins that activate adenylyl cyclase. The resulting increase in cAMP tends to inhibit voltage-

independent K^+ channels in the receptor plasma membrane and increase the uptake of Ca^{++} from the extracellular fluid. Artificial sweeteners act on different guanosine triphosphate-binding protein-linked receptors whose α subunits activate phospholipase C, leading to the release of calcium from internal stores. The increase in cytosolic calcium has a depolarizing effect on the taste receptor cells.

Bitter substances bind to T2R receptors, a large class of G protein-linked receptors.^{81,88} The α subunit of the gustducin trimeric guanosine triphosphate-binding protein activates phosphodiesterase, thereby reducing the level of cAMP. The reduction of cAMP removes the inhibition of outward K^+ movement. The $\gamma\delta$ subunit of gustducin activates phospholipase C β 2, leading to an increase in inositol triphosphate and the release of calcium from intracellular stores. Both the decrease in outward flow of K^+ and the increase in cytosolic calcium ions have a depolarizing effect on the taste receptor cells.

The multiplicity of bitter taste receptors—24 genes code T2Rs in humans—has evolved to avoid the ingestion of the wide variety of toxic chemical compounds that exist in nature. In contrast, sweet, umami, and salt are transduced by a narrow assortment of receptors and pathways, reflecting the restricted chemical and ionic species common to many nutritive foods.

Glutamate and other amino acids are responsible for the delicious (umami) taste common to meats, fish, and vegetable proteins. These stimuli are transduced by T1R1 and T1R3 metabotropic receptors.⁸⁷ Activation of the umami receptor leads to activation of phosphodiesterase and a reduction in cAMP. For nearly 100 years, Japanese and Chinese cooks have used monosodium glutamate to enhance the delicious (umami) flavor of their recipes.

Individual taste receptor cells do not appear to be specialized to respond to a single taste modality, nor are they equally sensitive to all five taste modalities. Intracellular recordings indicate that, although most taste receptor cells are sensitive to more than one taste modality, they respond most strongly to substances of one taste category. The existence of taste fields, for example, sweetness nearer the tip of the tongue and bitterness toward the back of the tongue, has been challenged by recent studies. Recent work suggests that T1R1-T1R3 heteromeric receptors for amino acids are more highly expressed anteriorly in fungiform papillae, while T1R2-T1R3 heteromeric receptors for sweet tastants are localized in circumvallate and foliate papillae located more toward the back of the mouth.^{87,90}

Neuronal influence on taste bud formation and maintenance

Taste buds degenerate after denervation and reappear after nerve regeneration.⁹¹ Disorganization and cellular degeneration begin within 2 days of nerve section, and taste buds are gone by the seventh day. Nerves that normally innervate taste buds, such as the chorda tympani, glossopharyngeal, and vagus nerves, can substitute for each other and cause buds to regenerate in any part of the tongue. Other sensory, motor, or autonomic nerves are ineffective.

Sensory neurons can maintain the buds independent of central nervous system connection. Experiments have shown that the sensory neuron is a pseudounipolar cell. It gives off a single axon that bifurcates into a peripheral process that innervates peripheral end-organs and a central process that connects it to the central nervous system. Cutting the central process of the sensory neuron does not harm the taste bud, while cutting the peripheral process leads to taste bud degeneration.

A number of recombination and reinnervation experiments have shown that neither a papilla nor its original epithelium is needed to form taste buds. The requirements seem to be oral epithelium plus the appropriate sensory neurons. Although the specific neurotrophic substances that are needed for the development and maintenance of taste buds in humans have yet to be identified, there is evidence that some taste receptors in mice are dependent on brain-derived neurotrophic factor for their survival.⁹²

The life span of an individual taste bud cell is believed to be only a few days. Thus, new cells must be constantly produced to maintain a viable taste bud. Conditions that interrupt cell renewal, for example local radiation, lead to atrophy of the taste buds.

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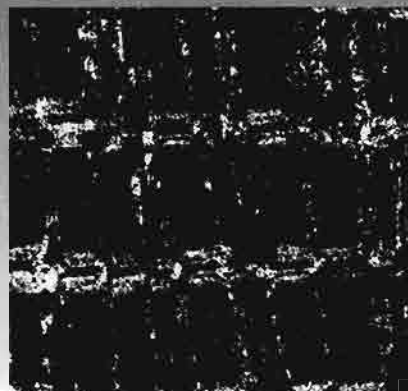
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Chapter 11

Muscle



Nearly every function performed by the mouth is dependent on the activity of contractile tissue. Mastication of food, swallowing, and speech require highly coordinated muscular action. In addition to these major physiologic functions, many subordinate functions essential to oral health, such as the regulation of vasomotor tone, the migration of various cell types, and the phagocytic function of neutrophils, involve contractile mechanisms performed at cellular and subcellular levels.

Muscles of Mastication

Jaw-closing muscles

In the mastication of food, the greatest expenditure of energy occurs during jaw closure. Many foods have to be crushed and triturated under high loading forces; thus, several relatively robust jaw-closing muscles are needed. The jaw-closing muscles include the temporalis, the masseter, and the internal pterygoid muscles¹ (Fig 11-1). The temporalis is a thin, fanlike muscle that arises from the temporal bone to converge to its attachment on the coronoid process and ramus of the mandible. It elevates and

retracts the mandible. The masseter arises from the zygomatic process and arch of the maxilla to insert into the mandibular ramus (see Fig 11-1). It raises and closes the jaw. The internal (medial) pterygoid arises from the medial surface of the pterygoid plate and palatine bone to insert on the medial surface of the mandibular ramus. It closes the jaw. The jaw-closing muscles are innervated by respective branches derived from the mandibular division of the trigeminal nerve.

Jaw-opening muscles

During the jaw-opening phase of the chewing cycle, minimal energy is needed. The external (lateral) pterygoid muscle runs horizontally from its origin on the infratemporal fossa to its insertion on the neck of the mandibular condyle (see Fig 11-1). It also attaches to the anterior segment of the articular disk. The external pterygoid muscle pulls the condyle forward and rotates it along the articular surface of the temporal fossa. It also stabilizes the articular disk during the rotation of the mandibular condyle. The initial phase of jaw opening is also helped by the actions of the mylohyoid, digastric, and stylohyoid muscles (see Fig 11-1).

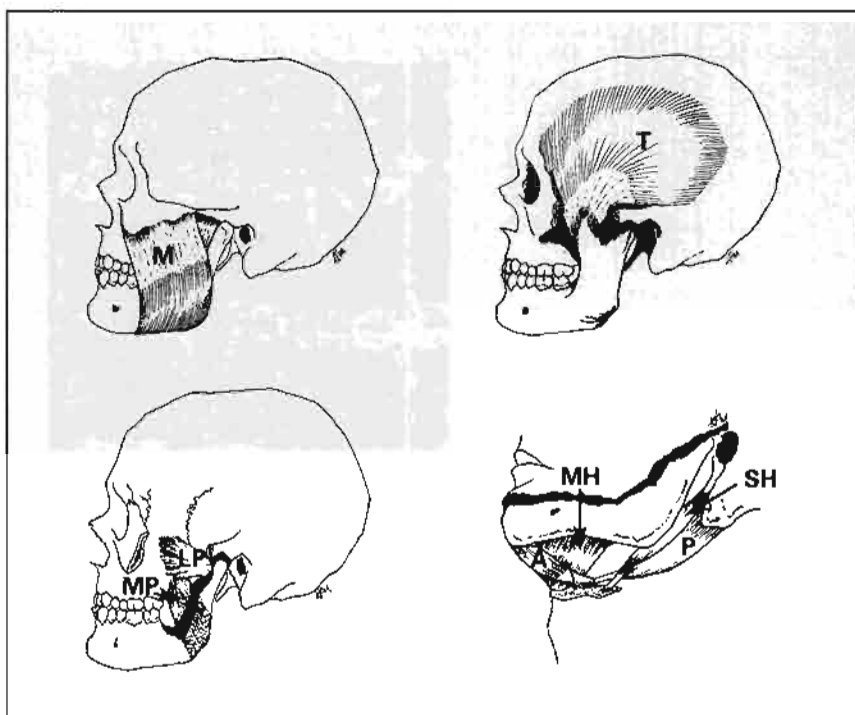


Fig 11-1 Muscles of mastication. The jaw-closing muscles consist of the masseter (M), temporalis (T), lateral pterygoid (LP), and medial pterygoid (MP) muscles. The jaw-opening muscles are the anterior (A) and posterior (P) bellies of the digastric muscle and the mylohyoid (MH) and stylohyoid (SH) muscles. (Reprinted from Mohi et al¹ with permission.)

Muscles of the Tongue, Soft Palate, and Pharynx

The tongue is a highly muscular organ capable of complex shape changes for handling food during mastication and subsequent swallowing. It is also extremely important in phonation, during which rapid and delicate muscular contractions of the intrinsic tongue muscles must be coordinated with muscles of the lip. There are four intrinsic muscle fiber groups: the superior longitudinal, inferior longitudinal, transverse, and vertical fibers. Intrinsic muscle fibers have origins and insertions in the submucosal connective tissues of the tongue. Intrinsic muscles accomplish shape changes of the tongue.

The extrinsic muscles carry out the extrusion and intrusion of the body of the tongue. The origins, insertions, and principal actions of the extrinsic muscles of the tongue, soft palate, and pharynx are described in detail in many textbooks of gross anatomy. These muscles participate in the complex functions of swallowing, breathing, and speaking and in reflexes such as coughing, vomiting, and sneezing.

Some of the aforementioned functions call for rapid muscular contractions, while others require

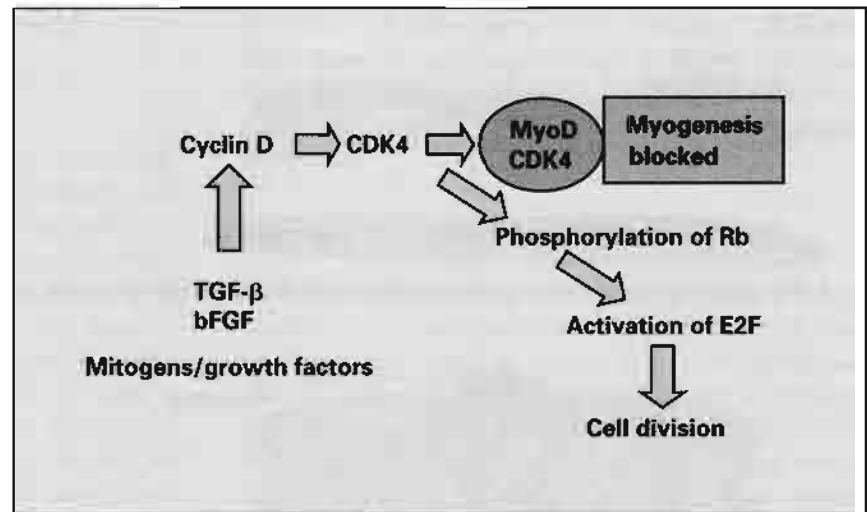
sustained contraction. Muscle fibers with different rates of contraction and relaxation, and different energy utilization pathways, evolved long before the appearance of vertebrates and have been retained in varying proportions in the muscles of the face and oral cavity.² For example, the palatopharyngeal and uvular muscles, which contract rapidly, are made up of a high proportion of type II muscle fibers, while the levator and tensor veli palatini, which contract more slowly, have mostly type I fibers.³ Muscle fiber types are discussed in a later section.

Development of Skeletal Muscle

In humans, myogenic cells of the paraxial mesoderm migrate and proliferate within the facial primordia between the 5th and 10th weeks of fetal life. Myotube formation occurs soon after mononuclear myoblasts align themselves toward bony sites of origin and insertion.

The development of the muscles of the head and neck have been best studied in the chick embryo.^{4,5} Development of these muscles begins during the 2nd week of embryonic life, with the appearance of the myogenic regulatory factor Myf5, shortly thereafter by

Fig 11-2 Cyclin D and cyclin-dependent kinase 4 (CDK4) block myogenesis by sequestering MyoD, a skeletal muscle-specific gene regulatory protein responsible for activating the synthesis of muscle proteins. Factors that increase cell proliferation, such as transforming growth factor β (TGF- β) and basic fibroblast growth factor (bFGF), block myogenesis by increasing cyclin D-CDK4 complexes. (E2F) A DNA transcription promoter needed for cell division; (Rb) retinoblastoma protein.



MyoD, and still later by myosin heavy chain proteins within cohorts of migrating myoblasts in the developing mesenchyme of the branchial arches. In general, the time course for the expression of these gene products is slower in the head and neck nonsegmental mesenchyme than in the trunk (somitic mesoderm) muscles. Myoblast differentiation in the head and neck requires initiating signals from adjacent migrating neural crest cells (and perhaps from the overlying epithelium), but these have yet to be identified.

In comparing the timing of the development of tongue muscles, Yamane et al⁶ reported that formation and maturation of myotubes progressed faster in the tongue than in the musculature of the hind limbs in mice. They suggested that the early completion of the tongue musculature might be needed for suckling soon after birth.

Skeletal muscle differentiation can only occur after myoblasts have exited the cell division cycle. Only then can the genes that transcribe muscle-specific proteins become activated. A key regulatory pathway involving MyoD, a transcription factor essential for skeletal myogenesis, and the proteins that control cell division, namely cyclin D and cyclin-dependent kinase 4 (CDK4) has recently been identified⁷ (Fig 11-2). MyoD is a member of the skeletal muscle-specific gene regulatory proteins (other members of this family include Myf5 and Myf4, or myogenin).

When MyoD is introduced into nonmuscle cells, it can direct them to differentiate into muscle cells. Although MyoD is present in myoblasts, it is in an inac-

tive form because of its sequestration by CDK4 (see Fig 11-2). Activation of CDK4 is dependent on the concentration of cyclin D, the major protein that drives cells through the G₁ phase of the cell cycle. Activated CDK4 (actually a complex of cyclin D and CDK4) also phosphorylates retinoblastoma protein, triggering the cell to enter the S phase of the cell cycle by releasing E2F transcription factor (see Fig 11-2).

Mitogens and growth factors that promote cell proliferation, such as transforming growth factor β (TGF- β) and basic fibroblast growth factor, do so by upregulating cyclin D levels and thereby blocking myogenesis. However, when mitogen and growth factor levels fall, cyclin D decreases, and the sequestration of MyoD by activated CDK4 is diminished. Under these conditions, MyoD causes the myoblast to enter the G₀ (quiescent) phase and begin terminal differentiation.

Another major regulator of myogenesis is the transcription factor myocyte enhancement factor (MEF2). Like MyoD, MEF2 activation is intimately connected to cell cycle regulatory factors. Cyclin-dependent kinase inhibitors and mitogen-activated protein kinase phosphatase participate in activating MEF2 by arresting the cell division cycle, thereby permitting myoblasts to undergo terminal differentiation.⁸ Once they have entered the G₀ state, myoblasts are able to fuse to form multinucleated myotubes (Fig 11-3).

Muscle-specific proteins begin to appear in the sarcoplasm shortly after myotubes have formed. As the contractile proteins increase in amount and be-

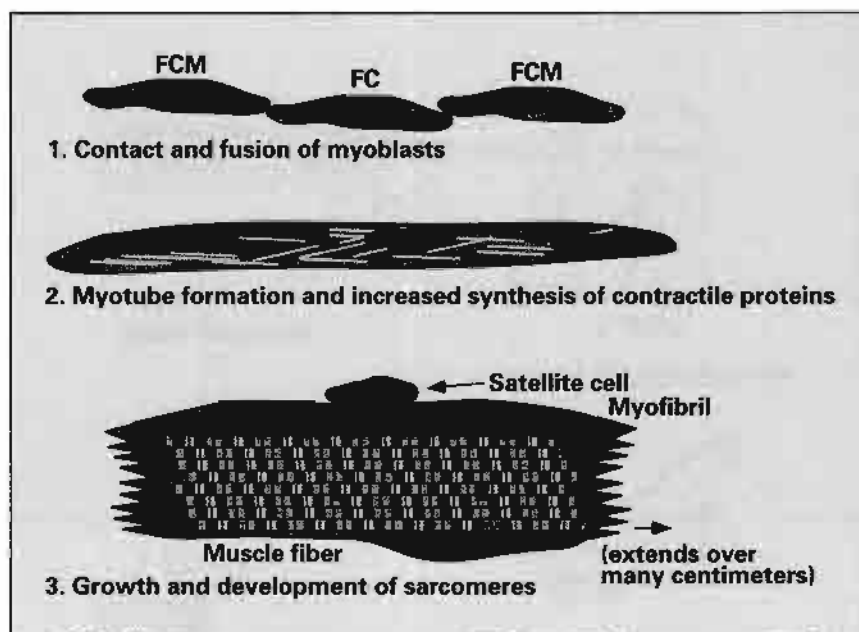


Fig 11-3 Development of skeletal muscle fiber. Skeletal myotube formation starts when founder cells (FCs) and fusion-competent myoblasts (FCMs) fuse. Myotubes grow by fusion of additional FCMs, and subsequently mature to form muscle fibers through synthesis and assembly of contractile and cytoskeletal proteins into sarcomeric subunits.

come assembled into strictly organized units called *sarcomeres*, the nuclei are pushed to the periphery along with other cytoplasmic organelles. A small number of myogenic stem cells persist in mature muscle in the form of satellite cells (see Fig 11-3).

Progress has been made in understanding the complex process of myoblast fusion through the study of myotube formation in the fruit fly, *Drosophila melanogaster*. Scientists have discovered two types of myoblasts in *Drosophila* muscle primordia, founder cells and fusion-competent myoblasts (FCMs).⁹ Founder cells have the special capacity to attract and fuse with FCMs. There are several genetically distinct founder cells (determined by the presence of identity genes). Each founder cell type appears to control the type of muscle fiber that eventually develops from fusion of FCMs.

Two key cell membrane proteins, Duf and Sns, members of the immunoglobulin superfamily, have been identified and found to be essential for fusion of myoblasts. Founder cells express Duf, a transmembrane protein that attracts FCMs. Contact of FCMs with the Duf-enriched surface of the founder cell initiates close contact and subsequent fusion of the opposing cell membranes (see Fig 11-3). A transmembrane protein on the FCM, Sns, appears to interact with the founder Duf protein during the recognition

and fusion process. Both proteins have sizeable cytoplasmic domains, suggesting that they may participate in signaling events essential to fusion.¹⁰

Fusion of additional myoblasts to the newly formed myotube proceeds from each end. The midregion of the developing muscle fiber prepares for the formation of a neuromuscular junction by interacting with signals from an approaching motor nerve ending. New sarcomeres continue to form as muscle fibers grow in length and width as individuals grow into adulthood.

The factors that regulate muscle size are complex and poorly understood. What is known is that muscles must perform work to maintain their mass. Prolonged bed rest, weightlessness of space flight, and paralysis cause muscle wasting. Disuse leads to sarcomeric breakdown and proteolysis of contractile proteins. In contrast, increased demand, such as repeated exercise, leads to increased muscle mass.

Muscle size is negatively regulated by myostatin, a newly discovered and highly conserved member of the TGF- β superfamily of growth regulators.¹¹ Abrogation of this protein causes a significant enlargement of muscle mass. A muscle-wasting complication in men infected with human immunodeficiency virus has been correlated to a circulating myostatin-like protein.¹²

Structure of Skeletal Muscle

Cell structure

Each muscle fiber is a large, elongated multinucleated cell. During the differentiation of myoblasts and the formation of mature striated muscle cells, numerous proteins of the actin and myosin systems are expressed at high levels. These proteins are assembled into sarcomeres. The sarcomeres are aligned end-to-end to form myofibrils. Hundreds of myofibrils, in parallel alignment, fill the bulk of the muscle cytoplasm (sarcoplasm) (see Fig 11-3). The nuclei and small Golgi apparatuses are restricted to the peripheral sarcoplasm. Mitochondria are distributed between the myofibrils and are also clustered in parts of the peripheral sarcoplasm.

Two specializations of the cell surface, the motor end plate and the transverse tubules (TTs), have evolved to maximize the rapid transfer of neural stimuli to all myofibrils. The motor end plate, a highly infolded domain of the muscle fiber directly opposite the motor nerve terminal, is described later, in the section discussing the neuromuscular junction. The TTs are connected to the plasma membrane (sarcolemma) via subsurface caveolae.¹³ The TTs conduct membrane depolarization, evoked by neurotransmitters at the motor end plate, deep in the interior of the muscle fiber. Transverse tubules communicate with the sarcoplasmic reticulum (SR), an adaptive specialization of the endoplasmic reticulum.

Muscle cells have developed a mechanism for increasing the integration of the sarcolemma, cytoskeleton, and myofibrils to the extracellular matrix (ECM).¹⁴⁻¹⁶ The dystroglycan and dystrophin proteins are key components of the integrating mechanism. Dystroglycan β , a transmembrane protein, binds laminin 2 of the basal lamina via the extracellular peripheral membrane protein dystroglycan α . Dystrophin is a peripheral cytoplasmic protein that bridges dystroglycan β to the actin cytoskeleton. Integrin receptors in the sarcolemma, along with their ligands in the basal lamina, contribute to the integration of the cytoskeleton to the ECM. The $\alpha 7$ integrin (laminin receptor) and the $\alpha 5$ integrin (fibronectin receptor) are the major integrin types found in mature muscle fibers.¹⁷

Integrins and components of the dystroglycan system are interconnected at myotendinous junctions, neuromuscular junctions, and at the costameres spaced along the sarcolemma.¹⁷ Costameres are specialized membrane domains that attach the Z-disk network to the ECM via the intermediate filament and

actin filament cytoskeleton.^{14,16,17} Mutations that lead to defects in the various components of the dystroglycan-dystrophin complex and the integrin receptors have been shown to cause various forms of muscular dystrophy.

Muscle fiber organization

A skeletal muscle is composed of numerous muscle fibers tightly integrated into a functional unit by connective tissue. Each muscle is enveloped by dense connective tissue, the epimysium. Sheets of connective tissue course inward from the epimysium to form the perimysium that surrounds subunits (fascicles) of the muscle. The perimysial connective tissue undergoes additional subdivision to form an endomysium surrounding each muscle fiber (Figs 11-4 and 11-5).

Skeletal muscle provides the contractile apparatus for moving rigid skeletal components in relation to one another. To accomplish this function, the muscle must be integrated to the bone surface by dense connective tissue at myotendinous junctions. At the origin and insertion of the muscle to the bone, the epimysial connective tissue assumes greater density as it merges to form a tendon (a cablelike attachment) or an external aponeurosis (a flattened sheet of connective tissue). Internal aponeuroses, dense bands of connective tissue within the muscle, provide internal origin and insertion points for fascicles, thereby creating functionally different subunits within a muscle.

The subunit or fascicular organization of a striated muscle permits a muscle to deliver a spectrum of diverse movements. For example, the masseter muscle of humans has a tripartite organization. The superficial part, containing two distinct bundles of muscle fibers, elevates and protrudes the mandible, while the deep portion, which also contains two bundles of muscle fibers, elevates and retrudes the mandible. The central and relatively homogenous part is involved in elevating the mandible.

Each fiber bundle may consist of numerous fascicles, oriented in parallel fashion and anchored either to a tendon or an aponeurosis. Muscles organized in this fashion are called *multipennate muscles*. The four masticatory muscles of humans (temporalis, masseter, internal pterygoid, and external pterygoid) are organized into 12 functional subunits, or fascicles, each having distinctly separate origins, insertions, and biomechanical functions.¹⁸

Serial section reconstruction of the rat medial pterygoid muscle reveals the complexity of its multipennate internal architecture.¹⁹ This muscle contains

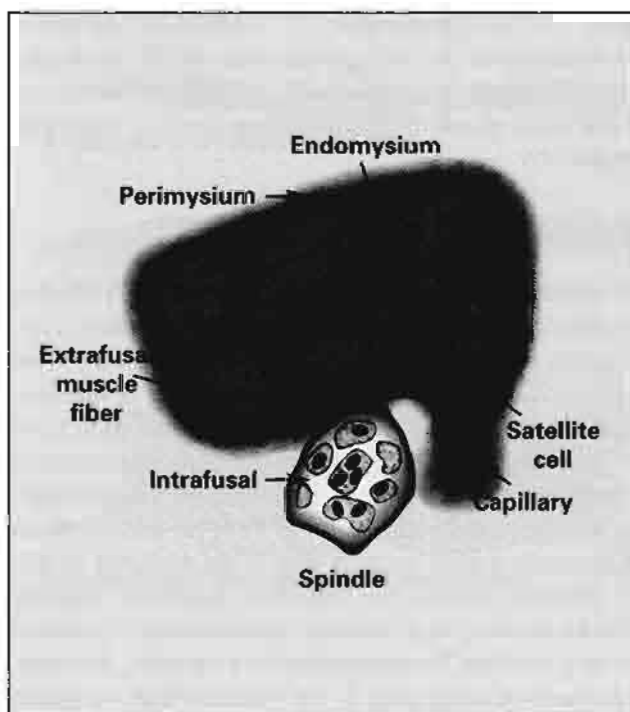


Fig 11-4 Muscles are compartmentalized by connective tissue components. Individual muscle fibers are surrounded by endomysial connective tissue. Groups or fascicles of muscle fibers, including spindles, are enveloped by perimysial connective tissue.

several internal aponeuroses and eight separate compartments (Fig 11-6). The masseter of the adult rabbit provides another example of a multipennate muscle. It contains 13 subdivisions, each with its own origins, insertions, and innervation.²⁰ The subunit structure of a multipennate muscle permits it to carry out several distinct actions, a quality essential to carrying out the complex tasks of mastication and swallowing.

In long fascicular muscles, such as the limb muscles and the sternomastoid, the individual muscle fibers do not extend from the origin to the insertion of the muscle. In these long series-fibered muscles, short intrafascicular muscle fibers are joined end to end by well-developed interdigitating adhesion junctions. These fiber-to-fiber (myomyonal) junctions have all the characteristic features found in the myotendinous junction.^{21,22} In addition to attachment by myomyonal junctions, adjacent muscle fibers are bound along their lateral surfaces by endomysial collagen fibers.



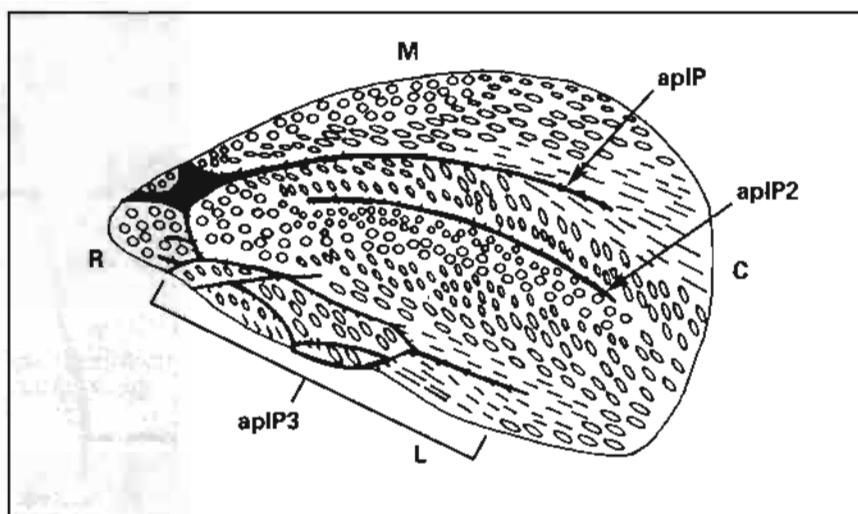
Fig 11-5 Thin section of tongue muscles containing transverse and vertical muscle fibers (MF). Perimysial connective tissue (Pm CT) surrounds muscle fascicles (Fasc) and merges with perineural and perivascular CT. (Cap) Capillaries; (NB) nerve bundle. (Toluidine blue stain. Original magnification $\times 240$.)

Integrin $\alpha 7$ subunits and dystrophin are key components of the myomyonal and the myotendinous junctions.¹⁷ The integrity of the internal connective tissue network and the myomyonal junctions is essential for transmission of tension from the muscle fibers to the extramuscular tissues.

Muscle fiber types

Another form of diversity within muscle, in addition to patterns of muscle fiber organization, relates to speed of contraction and manner of utilizing energy. Classically, two basic types of muscle fibers are recognized: type I, slow-contracting and fatigue-resistant red fibers (slow-twitch); and type II, fast-contracting and fatigue-sensitive white fibers (fast-twitch). Red fibers have a relatively high level of succinic dehydrogenase and numerous mitochondria, and they make greater use of the oxidative pathway over the glycolytic pathway. Red muscles also have a higher concentration of myoglobin and a richer supply of capillaries.

Fig 11-6 Diagram of a section through the rat medial pterygoid muscle illustrates its multipennate organization. Internal aponeuroses (black bands) compartmentalize the muscle fibers (circles) into several functional units. (aplP) Internal aponeuroses; (C) caudal; (L) lateral; (M) mesial; (R) rostral. (Adapted from Matsumoto and Katsura¹⁹ with permission from Elsevier Science.)



In contrast, white fibers have high adenosine triphosphatase (ATPase) activity, low succinic dehydrogenase activity, and a well-developed sarcoplasmic reticulum. They depend more on glycolytic utilization of glycogen stores, rather than the oxidative pathway for their energy needs. Type II fibers are classically subdivided into type IIa, fast-twitch, fatigue-resistant fibers, and type IIb, fast-twitch, fatigable fibers.

Rates of contraction and relaxation are the result of differences in expression of the many isoforms of myosin II, tropomyosin, troponins, and calcium-sequestering enzymes in the SR. In addition to differences in energy utilization pathways, fast-twitch muscles contract and relax more rapidly because of intrinsic differences in myosin ATPase activity.⁵ Although all muscles of mastication contain a mixture of these fiber types, the slowly contracting red fibers make up only a very small percentage of masticatory muscle fibers.²³

With the advent of more sophisticated analysis of muscle fibers, it has been shown that fast-twitch and slow-twitch muscle fiber types can be further subdivided into at least 11 categories based on the expression of different myosin heavy chain isoforms.²⁴ There are also different isoforms of myosin light chains, troponins, tropomyosins, calmodulin-dependent kinases, and SR calcium-binding proteins, all of which create a wide spectrum of physiologic response characteristics among fiber types. It has also become ap-

parent that neuromuscular activity, hormonal stimulation, mechanical loading, and aging can lead to reversible transitions between fiber types.²⁴ In summary, muscle fiber type and type stability are much more complex issues than was heretofore recognized.

Satellite cells

Adult muscle contains a pluripotent line of stem cells (called *side population cells*) that can give rise to most types of bloodborne cells and to satellite cells.²⁵ Expression of the Pax7 transcription factor appears to be a key event in converting the pluripotent side population stem cell into a more restricted satellite cell.²⁶ Satellite cells are self-renewing mononuclear cells that remain in a dormant state adjacent to mature muscle fibers. They are located within the borders of the muscle fiber basal lamina and the sarcolemma.

Satellite cells proliferate to give rise to new myoblasts when stimulated by the demands of exercise or during the repair of injured muscle fibers. The expression of muscle differentiation factors such as MyoD, Myf5, and myogenin announces the differentiation of a satellite cell daughter cell into a myoblast.

The growth factors and signaling pathways that trigger the proliferation of satellite cells and the subsequent formation of myoblasts are just beginning to be identified.²⁵ Insulin-like growth factor and members of the fibroblast growth factor family stimulate satellite cell proliferation, helped by hepatocyte

growth factor's ability to upregulate the expression of fibroblast growth factor receptors.^{25,27} Transforming growth factor β inhibits cell proliferation, satellite cell differentiation, and muscle growth.

A gene encoding a neutrophil chemokine (*LIX*) and a second gene encoding a messenger ribonucleic acid (mRNA)-binding protein involved in regulating chemokines are expressed in satellite cell within hours after muscle injury.²⁸ Based on this finding, it has been suggested that satellite cells may provide signaling molecules to coordinate tissue remodeling during postinjury muscle repair, in addition to their primary role of generating myoblasts.

Muscle spindles

To accomplish the coordinated movements of the jaw, the muscles of mastication must receive constant feedback of the position of the mandible relative to the maxilla of the cranial skeleton. This feedback information originates in several types of sensory end organs. Muscle spindles in the muscle mass, Golgi tendon organs located in the tendons of muscle insertions, and slowly adapting Ruffini-like nerve endings in joint capsules and the periodontal ligament send information back to the trigeminal sensory nuclei of the brain stem. The incoming signals are transmitted by monosynaptic and/or polysynaptic pathways to the motor neurons of the muscles of mastication.

The muscle spindle is a slowly adapting stretch receptor consisting of an assembly of miniature muscle fibers located within a muscle fascicle. The spindle relays information on the contractile status of its parent muscle back to the innervating α motor neuron in the brain stem. The muscle fibers of the spindle are known as *intrafusal fibers* and the fibers of the parent muscle as *extrafusal fibers* (see Figs 11-4 and 11-6).

Muscle spindles are found in small clusters within the middle or belly of a muscle, but no spindles are located near the origin or insertion of the muscle. Of the jaw-closing muscles, the masseter contains the greatest number of spindles.²⁹ Only a few have been reported in the jaw-opening muscles.

Each spindle contains several intrafusal fibers surrounded by a connective tissue capsule. There are two types of intrafusal fibers: small nuclear chain fibers characterized by a chainlike distribution of nuclei and large nuclear bag fibers identified by nuclei clustered within a middle expanse of sarcoplasm (Fig 11-7). Both types of intrafusal fiber are attached via a connective tissue capsule to the extrafusal fibers.

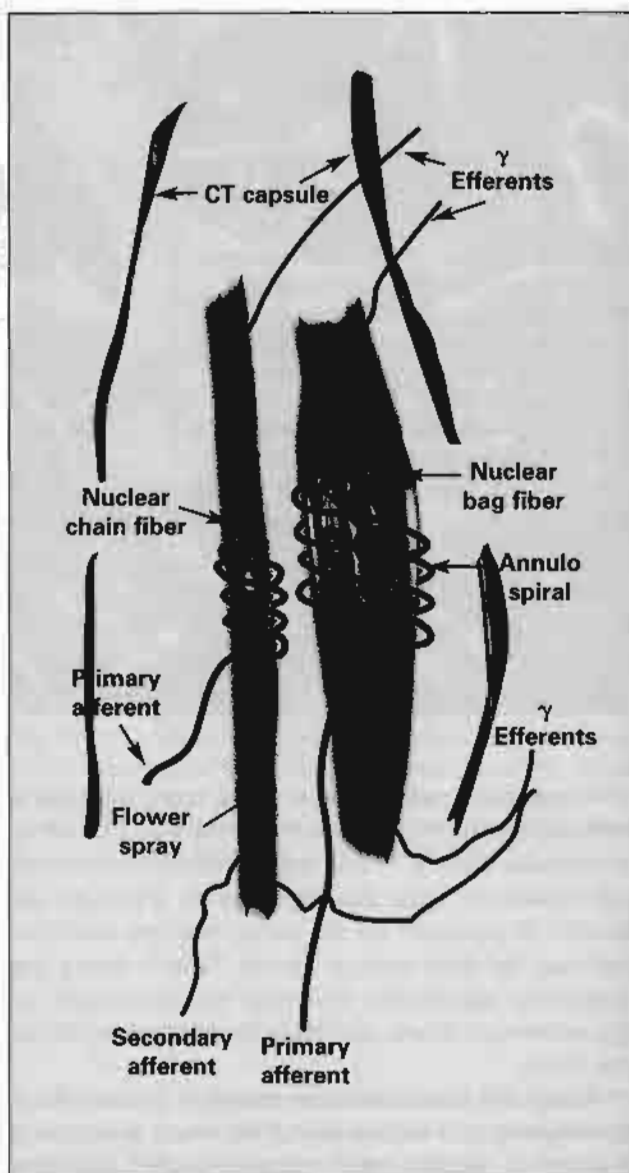


Fig 11-7 Muscle spindles contain nuclear chain and nuclear bag fibers. Each fiber is innervated by branches of primary and secondary afferent and γ -efferent nerves. (CT) Connective tissue.

The degree of stretching or contraction in the extrafusal muscle fibers is transmitted through the connective tissue to the intrafusal fibers and their sensory nerve endings. Each intrafusal fiber is innervated by a large type Ia myelinated nerve fiber (primary fiber) terminating in an annulospiral ending over the nucleated zone and by smaller myelinated fibers (secondary fibers) that terminate over the tapering ends in a flower-spray pattern of nerve endings (see Fig 11-7). The

primary nerves conduct information relating to the degree and rate of stretch, while secondary nerves relay information about the degree of stretch. A detailed description of the physiologic characteristics and the brain stem topography of primary and secondary spindle afferents of jaw muscles can be found in the work of Dessem et al.³⁰

Rapid stretching of a muscle spindle leads to a monosynaptic stimulation of the α motor neurons and a reflex contraction of the parent muscle. Fast-conducting primary fibers are responsible for the afferent limb of the stretch reflex. The reflex also activates a polysynaptic pathway for the inhibition of antagonistic muscles. In contrast to the stretch reflex, sudden unloading of a muscle causes rapid shortening of spindles and reflex activation of the antagonists.³¹

Intrafusal fibers also receive their own efferent input from γ motor neurons (see Fig 11-7). The γ motor neurons regulate the degree of contraction of the intrafusal fibers, thereby regulating the sensitivity of the spindles and, by reflex circuitry, the degree of tension in the extrafusal fibers. Contraction of intrafusal fibers, as a result of increased γ -efferent input, tends to stretch the midregion of the intrafusal fibers, activating annulospiral primary afferent nerve endings. Input along the primary afferents creates a stimulus to the α motoneurons in the brain stem, leading to increased contraction (tension) in the extrafusal fibers. This spindle-driven tension in the muscle is called α - γ coactivation.

In addition, α motoneurons receive proprioceptive feedback from the Golgi tendon organs, periodontal ligament mechanoreceptors, and various sensory nerve endings throughout the oral mucosal tissues. Proprioceptive feedback is essential in coordinating the muscular contraction used in chewing and mastication of food, while simultaneously protecting the associated mucosal tissues from injury.

Neuromuscular junction

Each motor neuron branches to innervate several muscle fibers to form a motor unit. During its development, a muscle fiber is contacted by several nerve axons, but as the muscle fiber matures, only one nerve ending gains dominance to form a mature motor end plate. The motor end plate, located in the midregion of the muscle fiber, is the site of synaptic contact with a nerve terminal of the α motor neuron. Motor end plates are located in a band running perpendicular to, and across, the muscle mass.²⁹

The motor end plate at the neuromuscular junction is the best studied of all synaptic contacts.^{32,33}

The architecture and the major components of the presynaptic and postsynaptic membranes are shown in Fig 11-8.

The Schwann cell sleeve terminates at the junction, thereby permitting the exposed nerve ending to make close contact with the muscle fiber. Only a basal lamina in a narrow synaptic cleft separates the bare nerve ending from the sarcolemma. The sarcolemma is highly infolded to provide a large surface area rich in nicotinic acetylcholine receptors (nAChRs) and acetylcholinesterase enzymes. During muscle development, the nAChRs are present at relatively low concentration along the entire sarcolemma; as the neuromuscular junction matures, nAChRs become concentrated in folds of the neuromuscular junction and are eliminated from all other parts of the sarcolemma (see Fig 11-8).

The basal lamina contains several proteins and proteoglycans that are essential for regulating and organizing the transmembrane components of the presynaptic and postsynaptic membranes. Agrin, a product secreted from the nerve ending, is localized in the basal lamina, where it plays a key role in clustering the nAChRs in the postsynaptic membrane. Agrin functions via a muscle-specific signaling kinase located in the sarcolemma.^{32,33} Rapsyn, a peripheral membrane protein, is also needed for clustering the nAChRs in the postsynaptic membrane.^{32,33} The nAChRs are concentrated in the crests of the folds of the postsynaptic membrane, and the voltage-gated Na^+ channels are concentrated in the troughs between the folds (see Fig 11-8).

Expression of the *nAChR* gene is stimulated by the signaling factor, neuregulin 1, which concentrates in the basal lamina following its release from the nerve ending (or from the muscle fiber, acting in an autocrine pathway).^{32,33} Neuregulin 1 acts as a ligand for Erb, a transmembrane tyrosine kinase in the postsynaptic membrane that activates *nAChR* gene transcription via phosphatidylinositol 3 kinase and the mitogen-activated protein kinase pathway.³⁴ The mRNAs needed for the translation of nAChRs are produced by a group of nuclei located in the immediate vicinity of the motor end plate.

Developing skeletal muscle nAChR is made up of five transmembrane proteins: (α 1)₂, β 1, γ , and δ . In adult nAChRs, the γ subunit is replaced by a δ subunit. The five proteins are arranged in a ring to form a central hydrophilic pore. In the closed state, the pore is occluded by a gate near the middle of the lipid bilayer formed by hydrophobic side chains of five leucine residues, one from each of the five transmembrane α helices. Extracellular domains

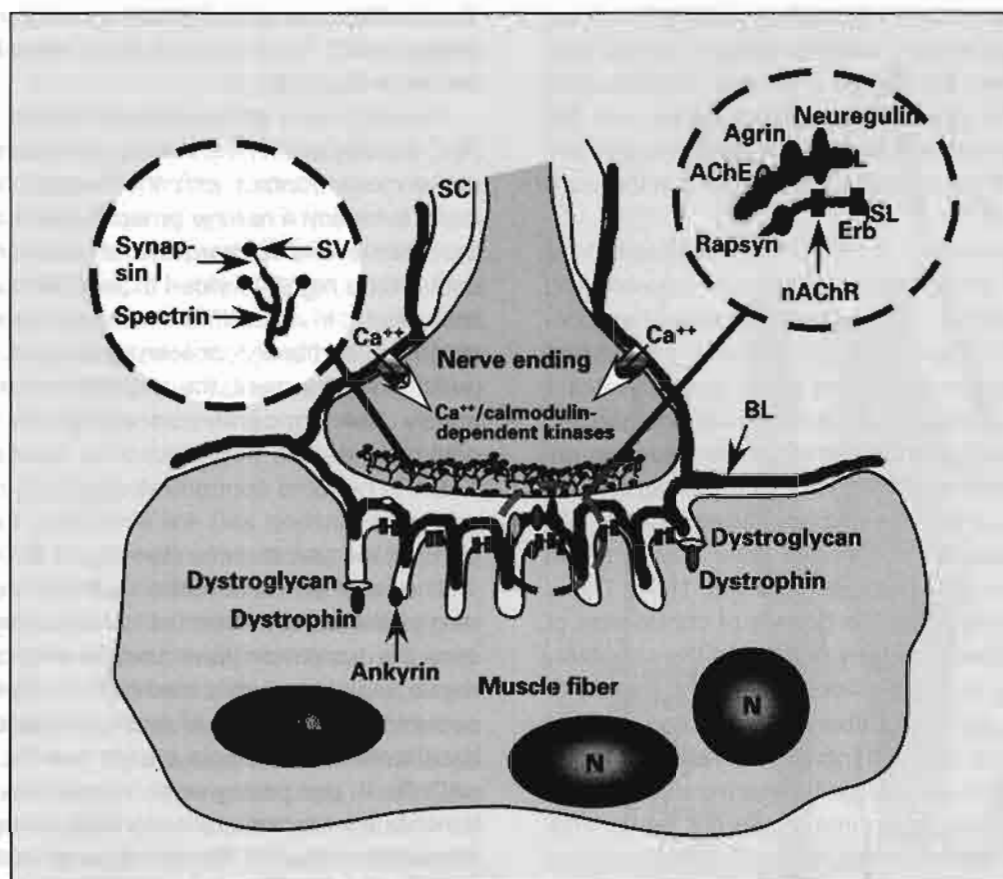


Fig 11-8 Architecture and major components of the neuromuscular junction. Coverage of the nerve by Schwann cells (SC) terminates proximal to the nerve ending, leaving only a basal lamina (BL) separating the nerve membrane from the muscle plasma membrane, or sarcolemma (SL). The BL acts as a scaffold for concentrating proteins (upper right inset) that organize and/or activate receptors in the adjacent SL. (Upper left inset) Two key proteins, spectrin and synapsin I, regulate the concentration of neurotransmitter synaptic vesicles (SV) at the nerve ending. (AChE) Acetylcholinesterase; (Erb) receptor tyrosine kinase for neuregulin; (N) nucleus; (nAChR) nicotinic acetylcholine receptor.

from the α , ϵ , and δ subunits form two acetylcholine-binding sites. When two acetylcholine molecules bind to the nAChR, the channel opens for about 1 millisecond and then closes. Subsequently, the acetylcholine dissociates from the receptor and is hydrolyzed by acetylcholinesterase.

Normally only Na^+ , K^+ , and some Ca^{2+} pass through the nAChR channel. Na^+ is favored by its high electrochemical gradient and by the fact that its concentration in the extracellular space is much higher than that of Ca^{2+} . About 30,000 ions of Na^+ go through each channel in 1 millisecond. The net effect is to depolarize the sarcolemma from about -60 mV to about -15 mV, a change sufficient to generate an action potential. The action potential spreads along the muscle

membrane by activation of voltage-gated Na^+ channels. The spread of the action potential into the transverse tubules triggers the release of calcium from the sarcoplasmic reticulum, causing the muscle to contract.

Myasthenia gravis is a neuromuscular disease caused by antibodies to nAChRs.³⁵ Oral complications in myasthenia gravis include poor masticatory performance and difficulty in swallowing.³⁶

Mastication

Stimulation of various peripheral somatosensory receptors can trigger reflex activation or inhibition in the muscles of mastication. A jaw-opening reflex can be

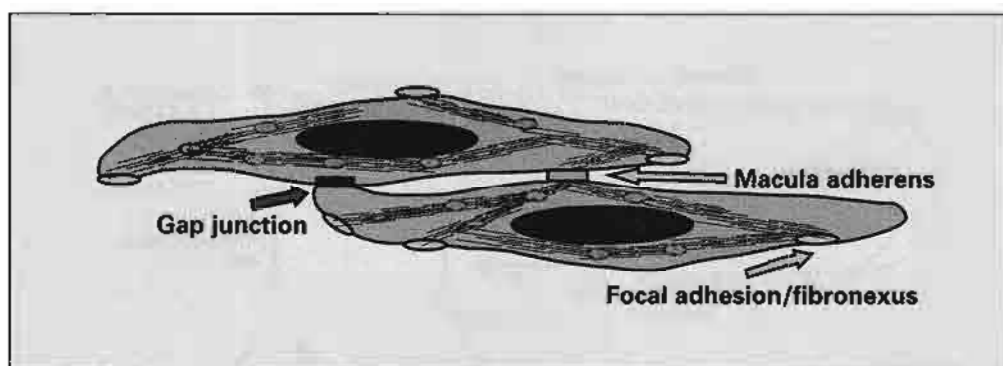


Fig 11-9 Smooth-muscle cell junctions. Smooth-muscle cells are functionally integrated by well-developed macula adherens junctions, gap junctions, and cell-to-matrix junctions of the fibronexus type. Adherens junctions and fibronexus junctions integrate the contractile filaments and the cytoskeleton to the extracellular matrix. Gap junctions integrate the cells electrically and metabolically.

elicited when a noxious stimulus is applied to the oral mucosa and/or the teeth. The jaws open when sensory inputs from peripheral nociceptors and mechanoreceptors reach the brain stem to cause a polysynaptic reflex contraction of the digastric muscle and a monosynaptic temporary inhibition of the jaw-closing muscles.³⁷ Activation of periodontal ligament mechanoreceptors leads to a reflex inhibition of the jaw-closing muscles followed by an excitatory response.³⁸

Early neurophysiologic studies of mastication placed emphasis on these and other reflexes as the main initiators of the cyclic muscular events of chewing. It has become clear, however, that chewing and swallowing are complex events that cannot be explained by simple reflex activities.

It is now known that muscular action during the mastication of food is initiated and its rhythmic nature is regulated by a central pattern generator located within the brain stem.^{39,40} The rhythmic pattern of muscle activity is set by interneurons that regulate the output of trigeminal motoneurons according to incoming signals from peripheral somatosensory receptors.⁴⁰ The pattern generator interneurons can be overridden by cortical centers as well as aborted by reflex input from peripheral sensory receptors located in the periodontal ligament, oral mucosa, temporomandibular joint, and muscle spindles.⁴¹

Recent work suggests that primary afferents whose cell bodies lie in the brain stem are intimately involved with the pattern generator.⁴¹ For example, noxious stimuli arising from the masseter can alter the rhythm of the pattern generator as part of a pain adaptation mechanism to decrease forceful contraction of the jaw-closing muscles.^{39,42}

Structure of Smooth Muscle

Smooth muscles are composed of numerous individual smooth-muscle cells specialized to act in concert to deliver tension to adjacent nonmuscular tissues. To accomplish this, the smooth-muscle cells must be joined by adhesive and communicating junctions (Fig 11-9). The major cytoplasmic constituents are bundles of actin and myosin II filaments, in a ratio of 12:1, arranged parallel to the long axis of the cell. Although there is no sarcomeric architecture, the actin and myosin II filaments are assembled to effect a sliding filament action on stimulation by calcium.⁴³

Smooth-muscle myosin II is similar to myosin of skeletal muscle in that each myosin molecule contains two heavy chains, each with a noncovalently bound regulatory light chain and an essential light chain.⁴⁴ A cytoskeleton of intermediate filaments (desmin in visceral smooth muscle and vimentin in vascular smooth muscle) provides a scaffold on which the actin and myosin II filaments are organized and stabilized.¹⁵ Dense bodies in the cytoplasm and dense bands located just beneath the cell surface contain α -actinin and other proteins that serve to connect the contractile fibers to the intermediate filament cytoskeleton.¹⁶ This interconnection of cytoskeleton, cell surface, and contractile fibers causes the smooth-muscle cell to change its shape during contraction, while simultaneously transmitting tractional force to the extracellular matrix and to adjacent smooth-muscle cells.

Three types of cell junctions, maculae adherens, fibronexus-like junctions, and gap junctions, are essential for normal function of smooth muscle (see Fig

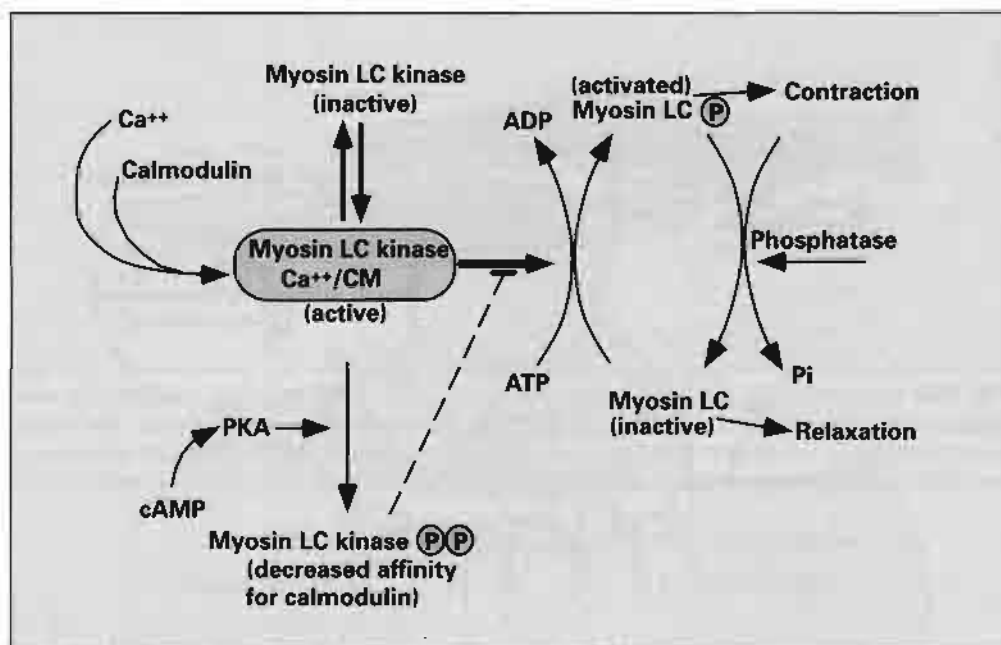


Fig 11-10 Calcium and cyclic adenosine monophosphate (cAMP) regulation of myosin light chain (LC) kinase. Contraction of smooth muscle occurs when myosin regulatory (LC) is phosphorylated. Myosin LC kinase is the major enzyme responsible for phosphorylating myosin. Because myosin LC kinase is a calcium- and calmodulin (CM)-dependent enzyme, the internal Ca^{++} concentration is the primary initiator of contraction. Relaxation is induced by cAMP (via protein kinase A [PKA]) and myosin LC phosphatase. (ADP) Adenosine diphosphate; (ATP) adenosine triphosphate; (P) phosphorylation; (Pi) inorganic phosphate.

11-9). Adherens junctions bind adjacent smooth-muscle cells across narrow intercellular spaces. The fibronexus is formed by patches of cell membrane rich in integrin receptors that bind extracellular matrix components such as fibronectin and collagen. Tension developed during contraction of the smooth-muscle cell is transmitted across the fibronexus to the extracellular matrix. Gap junctions function as electrotonic synapses to spread depolarization among adjacent smooth-muscle cells, thereby permitting the smooth-muscle cell mass to function as a syncytium.

Each smooth-muscle cell is an elongated, spindle-shaped cell with a centrally placed nucleus. The plasma membrane is characterized by many small invaginations or caveolae. These patches of caveolar membrane contain calcium pumps (Ca^{++} -ATPase), suggesting that they may serve as sites of calcium concentration and transport somewhat analogous to the sarcoplasmic reticulum of skeletal muscle. The cytoplasm contains numerous mitochondria, small Golgi apparatuses, smooth endoplasmic reticulum, and rough endoplasmic reticulum.

Each smooth-muscle cell is surrounded by a basal lamina containing laminin, fibronectin, entactin, and

collagen type IV. Collagen and elastase are secreted in small amounts to form a pericellular extracellular matrix. Blood vessels and nerves course within the wider connective tissue compartments of the smooth muscle.

Smooth-muscle contraction

The ATPase activity of myosin II of smooth muscle is regulated in a fundamentally different way than it is in skeletal and cardiac muscle. In smooth muscle, the stimulatory effect of calcium ion is mediated through calmodulin and the activation of myosin light chain kinase (Fig 11-10).⁴⁵ Activated light chain kinase phosphorylates myosin regulatory light chain, a prerequisite event that permits the globular motor domain of myosin heavy chain to contact actin, resulting in an ATPase-driven conformational change (filament slide). The globular motor domain of smooth-muscle myosin has different kinetic properties from that of skeletal muscle. In addition, smooth-muscle myosin has a longer contact time with actin, and greater force is generated per hydrolytic cycle.⁴⁴ In contrast, relaxation of smooth muscle is caused by dephosphoryla-

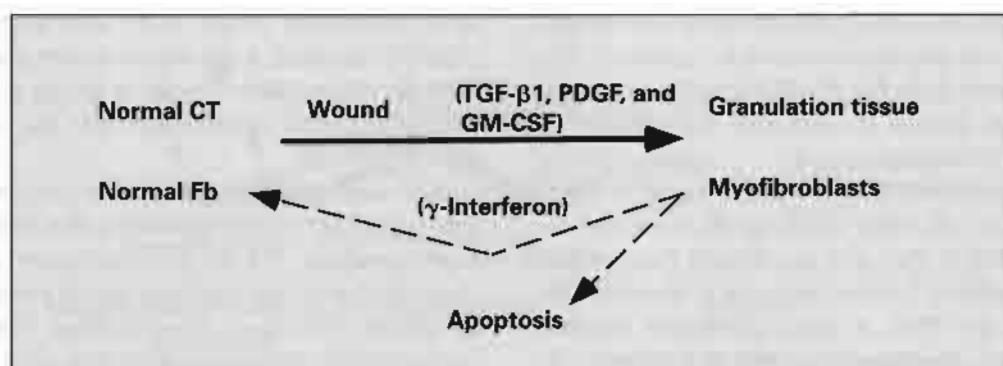


Fig 11-11 Role of growth factors and cytokines in regulating myofibroblast development and regression. (CT) Connective tissue; (Fb) fibroblast; (GM-CSF) granulocyte-macrophage colony-stimulating factor; (PDGF) platelet-derived growth factor; (TGF- β 1) transforming growth factor β 1.

tion of myosin light chain by myosin light chain phosphatase (see Fig 11-10).⁴⁵

The contractile apparatus is also regulated by caldesmon and calponin, two actin-associated proteins that inhibit the ability of myosin to hydrolyse adenosine triphosphate (ATP). Phosphorylation of caldesmon and calponin removes their inhibitory effect.

Second messengers generated in various signaling systems regulate smooth-muscle contraction by interacting with the calmodulin activation pathway or the phosphatase-inactivating pathway. For example, cyclic adenosine monophosphate (cAMP), via protein kinase A, deactivates myosin light chain kinase by a double phosphorylation of the protein (see Fig 11-10).⁴⁶ In the phosphorylated state, myosin light chain kinase has a lower affinity for the Ca^{++} -calmodulin complex and is therefore inactivated. In contrast, a pathway leading to contraction of smooth muscle and stress fibers involves inactivation of myosin light chain phosphatase via diacylglycerol and protein kinase C.⁴⁶ These signaling pathways tend to alter or set the tone of the smooth-muscle contraction.

The same mechanisms regulate the contraction of stress fibers in various cell types. The contraction of filament systems that are anchored to attachment plaques in the plasma membrane, such as those that form part of terminal webs, also appear to be under similar controls. This is illustrated by the action of Ca^{++} and cAMP second messengers in controlling the width of the lateral intercellular space between endothelial cells. Substances that trigger the release of Ca^{++} from intracellular stores contract endothelial cells during diapedesis of leukocytes. In contrast, agents that increase the production of cAMP cause contractile systems to relax, resulting in closure of the intercellular gaps.

Myofibroblasts

Myofibroblasts are found in granulation tissue and wound-healing sites. They are characterized by well-developed contractile stress fibers (SFs), basal lamina-like material concentrated along the external surface of the plasma membrane, and many gap junction interconnections with adjacent myofibroblasts.⁴⁷ Myofibroblasts express smooth-muscle myosin II, smooth-muscle- α -actin (SMA), and desmin. Stress fibers are bundles of SMA and myosin II filaments oriented parallel to the long axis of the cell.

Myofibroblasts synthesize and secrete ECM that is rich in collagen and fibronectin, and exert tension on the ECM through contraction of their stress fibers. If the ECM is not bound to stable structures, the action of the myofibroblasts will cause it to contract. Although normal fibroblasts have the ability to contract extracellular matrices, myofibroblasts are able to generate greater force.⁴⁸

Myofibroblasts are prominent in the later stages of wound healing, in hypertrophic scars, burn contractions, proliferative fibromas, and in some stromal responses to neoplastic lesions. A more fundamental role for fibroblast stress fibers may be to regulate the orientation of extracellular collagen bundles.⁴⁹ This is thought to occur by the interaction of contracting SFs, integrins, and fibronectin fibrils at the cell surface.

The differentiation of myofibroblasts is regulated by several growth factors and chemokines (Fig 11-11). Granulocyte-macrophage colony-stimulating factor and TGF- β 1 stimulate granulation tissue formation with increased numbers of myofibroblasts.⁵⁰ An outside-in integrin-signaling pathway, activated by extracellular fibronectin, is required for the TGF- β 1 induction of smooth-muscle actin in developing myofi-

broblasts.⁵¹ Platelet-derived growth factor and fibroblast growth factor also stimulate the formation of SFs in myofibroblasts (see Fig 11-11).⁵² Interferon γ acts in an opposite fashion to decrease the number of SFs and scar formation.

A CXC chemokine, chicken chemotactic and angiogenic factor, has been found to stimulate the expression of SMA in vitro and to increase the number of myofibroblasts in healing wounds.⁵³ Myosin light chain kinase and Rho, a small guanosine triphosphatase, induce the assembly of stress fibers in fibroblasts and myofibroblasts.⁵⁴ Endothelial cells can stimulate myoepithelial cell development through a paracrine pathway involving secretion of endothelin.⁵⁵

In vitro studies have shown that fibroblasts growing on mechanically stressed collagen substrates develop SFs and exert tension on the ECM.⁵⁶ The periodontal ligament fibroblasts of the transseptal fibers contain SFs, suggesting they may also exert tension on the collagen fibers.⁵⁷ Fibroblasts cultured from various organs exhibit different proportions of SMA-positive cells and α -SMA-negative cells.⁵⁸ The α -SMA-positive cells show larger adhesive contacts and stress fibers, and more mobility, than do their α -SMA-negative counterparts. Dugina et al⁵⁸ suggested that there might be at least two subsets of fibroblasts with different functions in most connective tissues.

Basic Science Correlations

Actin system

Conventional actin and a large family of actin-associated proteins (ARPs) are present in all cells.⁵⁹ There are six isoforms (with 80% homology) of conventional actin. Diversity among these isoforms resides in their amino terminals. Actin is present in both monomer (globular [G-actin]) and polymer (filamentous [F-actin]) forms. Each G-actin molecule has four binding sites: two for the formation of linear polymers of F-actin and two that can form interfibrillar bonds.

Actin filaments are constructed by end-to-end polymerization of G-actin. Each filament is made up of two chains helically intertwined (Fig 11-12). In electron micrographs, these filaments (microfilaments) are observed to be about 8 nm in diameter. They are present in relatively high numbers just beneath the cell membrane in the cortical cytoplasm.

To provide the energy needed for the polymerization step, G-actin must bind ATP and hydrolyze it to adenosine diphosphate (ADP). The polymerized

actin molecules retain ADP, and inorganic phosphate is released. Actin filaments are dynamic polarized structures. New G-actin is added at the positive end and G-actin is removed from the negative end (see Fig 11-12).

Stabilization of individual F-actin polymers requires interaction with capping proteins that have actin-binding properties. F-actin polymerization and depolymerization are essential features of a wide spectrum of cellular functions. Phagocytosis, cell migration, cell-substrate adhesion, cell division, and osteoclastic bone resorption are just a few of the cellular activities that require assembly of F-actin networks. Polymerization of F-actin occurs following the exposure (uncapping) of the positive end of an existing filament or it can be initiated *de novo* through the action of the recently discovered ARP2/3 complex.

Actin-rich cortical cytoplasm has a gel-like property when a high proportion of actin is in the filamentous form. Additional proteins that contain actin-binding domains, such as spectrin and filamin, add to the gelatinous property of actin networks by capping and stabilizing F-actin. When much of the actin is present in globular form, the cytoplasm has a sol or fluid nature. Gel-to-sol, and sol-to-gel, transitions are regulated by numerous factors, including the local concentration of calcium ions, cAMP, and ATP. Gelsolin, a Ca^{++} -dependent enzyme, severs actin filaments and caps the newly created fast-growing ends (see Fig 11-12).^{60,61}

Every cell contains a molecular tool chest filled with the tools needed to bind, polymerize, cap, cleave, and transport actin molecules. Profilin is a G-actin-binding protein that causes G-actin to exchange adenosine diphosphate for ATP, thus promoting filament assembly. Profilin-actin dimers can also act as a reservoir of monomers until a barbed end is exposed or a nucleating site (ARP2/3) becomes activated.

Rapid actin filament formation is responsible for cell migration, cytoplasmic contraction, and the protrusion of cell processes.⁶²⁻⁶⁴ For example, in the formation of filopodia and lamellipodia of migrating cells, F-actin filaments polymerize at a 55-degree angle to the plasma membrane, thereby creating the force needed to push the membrane outward.^{65,66} In this process, G-actin is rapidly added to the growing positive end of the filament.

A seven-protein complex of actin-associated proteins (ARP2/3) has a central role in organizing actin meshworks that initiate and drive cytoplasmic protrusion in migrating cells.^{64,67} The ARP2/3 complex is localized at the leading edge of cells, where it induces

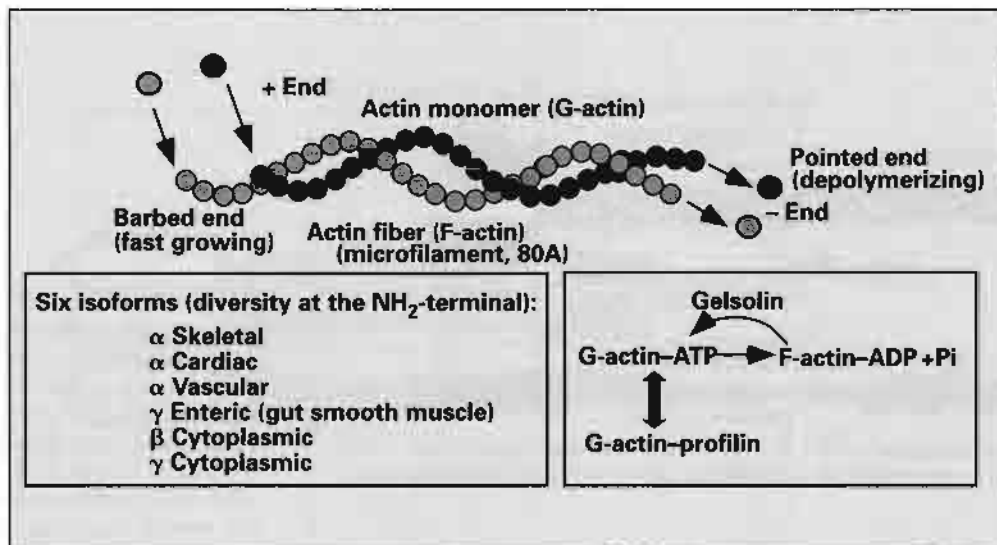


Fig 11-12 Actin filament formation and regulation by various G-actin-binding proteins and F-actin-severing proteins. (ADP) Adenosine diphosphate; (ATP) adenosine triphosphate; (Pi) inorganic phosphate.

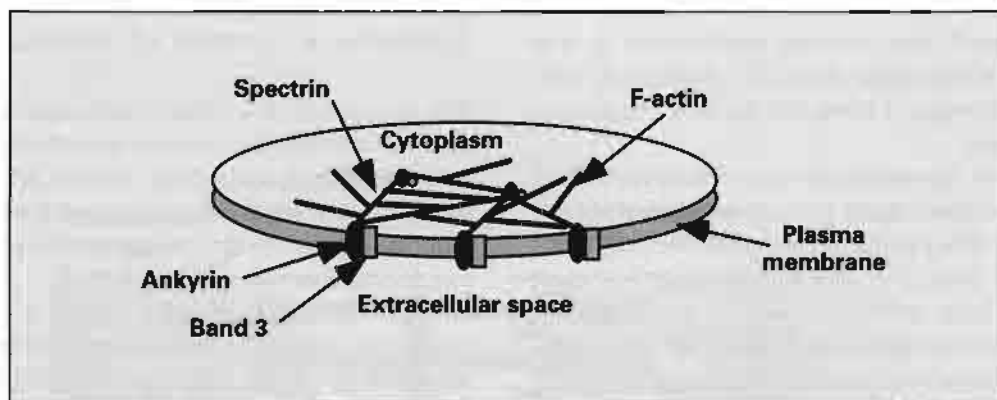


Fig 11-13 Spectrin cortical cytoskeleton. Spectrin serves as a bridge between the integral membrane proteins, ankyrin and band 3, and the subsurface actin filament network.

actin filament nucleation. Actin dimers elongate while capped at their negative (slow-growing) ends by ARP2/3. New actin filaments nucleate off the sides of older actin filaments by ARP2/3 in a dendritic pattern, driving the protrusion of cytoplasmic lamellipodia.^{65,66} The involvement of ARP2/3 in actin filament assembly is needed for phagocytosis of bacteria opsonized with Fc and complement C3 fragments.⁶⁸ Actin filaments nearer to the cell body are severed by cofilin and/or gelsolin to provide actin monomers for filament growth in the subcortical cytoplasm.

Cofilin is an actin-binding protein that depolymerizes actin filaments.⁶⁹ One mechanism for regulation of actin polymerization is through the phosphorylation of cofilin by serine kinases. In the phosphorylated state, cofilin is inactive and actin filament net-

works are stabilized.⁶⁹ Gelsolin activity has been shown to be necessary for normal motility of several cell types, including gingival fibroblasts.

The spectrin system is a group of specialized transmembrane and cortical cytoplasmic proteins that provide support for the cell membrane. It was first described in red blood cells but is now known to exist in some degree in all cells. The major components include spectrin, band 3 protein, and ankyrin (Fig 11-13). Spectrin is a member of a family of proteins that includes α-actinin of the Z disk and dystrophin of the subsarcolemmal cytoskeleton. Ankyrin and band 3 are transmembrane proteins serving to attach the spectrin network to the cell membrane. The F-actin filaments attach to the actin-binding domains of spectrin, thereby forming a latticework of actin-spectrin attached to the

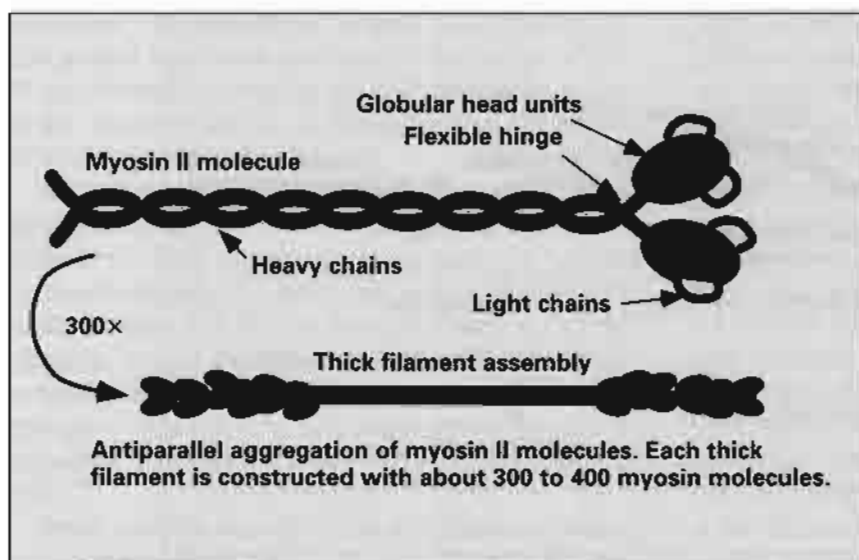


Fig 11-14 Domains of myosin II. The myosin II molecule consists of two myosin II polypeptides, each with a long heavy chain (tail domain) and a globular head (the adenosine triphosphatase enzymatic domain). Antiparallel alignment of the tail domains of hundreds of myosin II molecules form the thick filaments of striated and smooth muscle as well as of stress fibers of myofibroblasts.

cell membrane.⁷⁰ This network participates in the maintenance of cell shape and in the distribution, mobility, and anchorage of other cell surface transmembrane molecules.

In addition to its association with the spectrin network, F-actin is attached to the cell membrane via association with other proteins, such as vinculin, talin, α -actinin, and integrins. These molecules are concentrated at focal adhesions and in the larger fibronexus attachments. The formation of focal adhesions also involves the recruitment of focal adhesion kinase to the cell membrane. Thus, signal transduction events accompany the formation of actin-rich attachment sites at the cell membrane.

The signal transduction events related to the attachment of cells to the substratum appear to have a fundamental role in cell differentiation and cell proliferation. For example, noncancerous cells will not proceed through phase G₁ to phase S unless attached to a substratum.

Although F-actin is a noncontractile molecule, it participates with myosin in forming a contractile apparatus that ranges from simple aggregates to more complex structures, such as stress fibers and myofibrils. The most highly ordered arrangement of actin is found in the sarcomeres of cardiac and skeletal muscle. Here six F-actin filaments are radially disposed in a parallel alignment around one myosin thick filament. The F-actin filaments are capped at their positive end by Cap Z protein and held in a regular lattice arrangement by attachment to α -actinin, a major component of the Z disk.

Sarcomeric system of skeletal muscle

The sarcomere of striated muscle represents the pinnacle of contractile filament organization and function.⁷¹ Myosin II, actin, titin, nebulin, and many other structural and regulatory proteins are assembled into a nearly crystalline structure capable of rapid repetitive shortening.

Type II myosin molecules contain a globular head group plus a long tail domain required for self-association to form myosin thick filaments (Fig 11-14). Type II myosin is present in all muscle cells and in lesser amounts in many other cell types. The type I myosin molecule is a more primitive globular molecule found in all cell types.⁷²

The myosin II molecule is made up of two heavy chains (200 kDa), coiled to form a helical tail, and two globular heads separated from the tail by a flexible hinge domain.⁵⁴ A pair of light chains is associated with each globular head. The ATPase activity of myosin resides in the globular or motor domain.

Thick myosin filaments are formed by aggregation of myosin molecules in an antiparallel association with the tail segments. There are about 300 to 400 myosin molecules in a single thick filament. The thick filaments are stabilized in the sarcomere by binding to the titin scaffold and to proteins of the M band. The antiparallel aggregation of myosin permits contraction of myofibrils as the F-actin thin filaments are pulled toward the M band by the action of the ATPase of the myosin. In the sarcomere of cardiac and skeletal muscle, a nearly crystalline arrangement of actin-

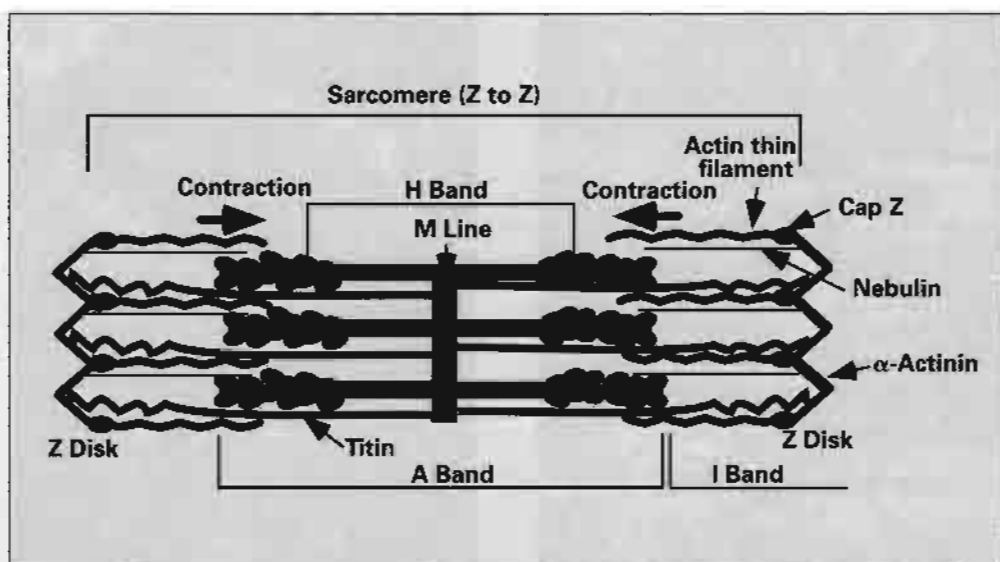


Fig 11-15 Arrangement of the major proteins of the sarcomere. Titin spans the distance from the M-line complex to the Z disk, thereby setting the length of the sarcomere. At its Z-disk terminal, the titin protein contains a domain with springlike properties. Actin filaments are supported by nebulin and are anchored to the Z disk by α -actinin.

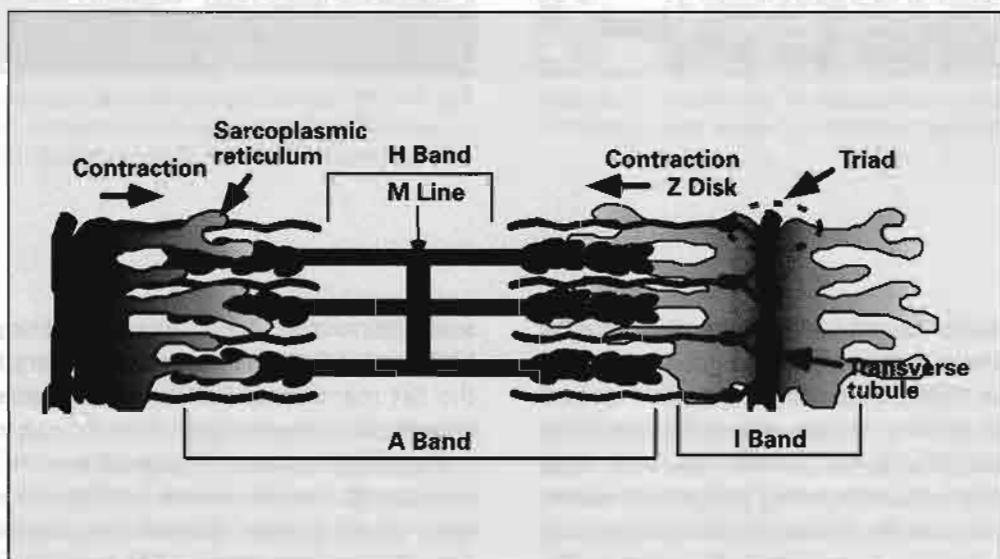


Fig 11-16 Location of the transverse tubules and saccules of the sarcoplasmic reticulum vis-à-vis the contractile elements of the sarcomere. The transverse tubules penetrate the interior of the striated muscle cell, where they associate with two segments of sarcoplasmic reticulum to form triads.

myosin is achieved. The sarcomeric organization of actin and myosin is regulated and maintained by numerous proteins that form a sarcomeric skeletal lattice (Figs 11-15 and 11-16).

Titin, at 1 μ m in length and with a mass of 4,200 kDa, is one of the largest proteins yet identified.⁷³ It spans half the length of the sarcomere, acting to bridge the Z disk to the M-line complex.⁷¹ Titin con-

tains two major segments, one in the A band, serving to stabilize the thick filaments and to attach to the M-line complex, and a second segment localized in the I band attached to the Z disk.⁷⁴ The I-band segment of titin possesses springlike properties that are (along with the compliance characteristics of myosin) essential to restoring the sarcomere back to its original length following contraction.^{73,74} Mutations in the titin

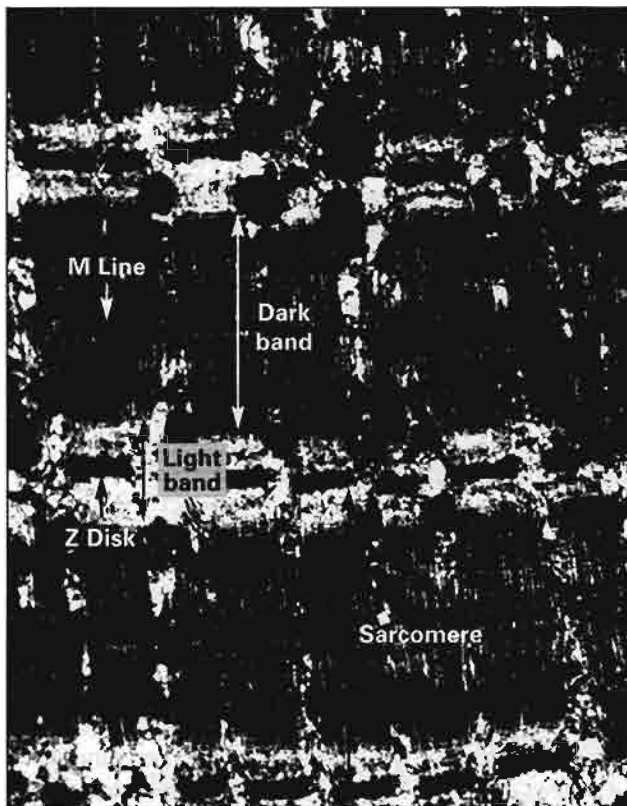


Fig 11-17a Electron micrograph of sarcomeres of striated muscles in longitudinal orientation. (Original magnification $\times 20,000$.)

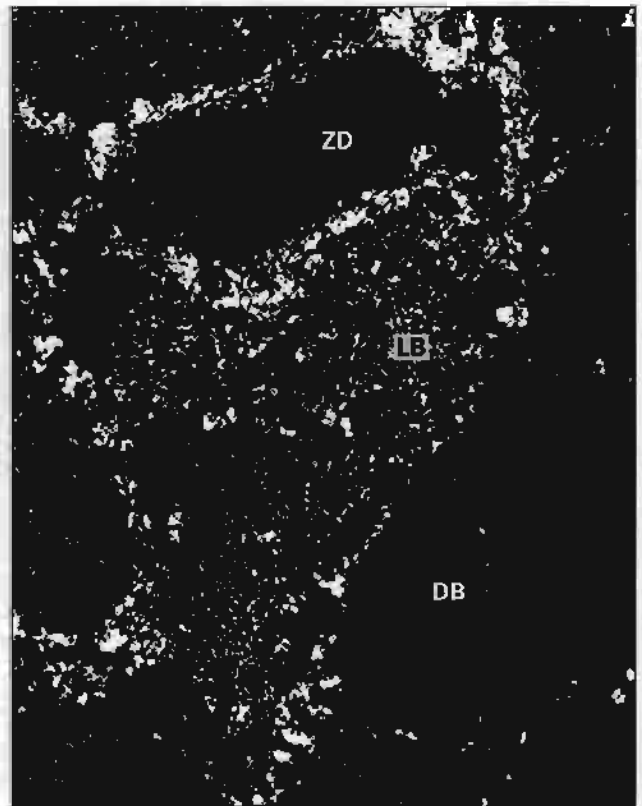


Fig 11-17b Higher magnification electron micrograph of sarcomeres of striated muscles in cross section. (DB) Dark band; (LB) light band; (ZD) Z disk. (Original magnification $\times 38,000$.)

protein have been associated with cardiomyopathy and heart failure in humans.⁷⁵

Nebulin is an 800-kDa actin-binding protein located in the I band of skeletal muscle, where it controls the length of the actin filaments. Cardiac muscle contains a smaller protein, nebulin, which performs a similar function. Several proteins, including α -actinin and Cap Z, control the binding and organization of thin filaments at the Z disk. In electron micrographs, the Z disk has the appearance of an electron-dense structure demarcating the extremities of each sarcomere (Fig 11-17). At least four proteins have been identified in the M band, where they help control the organization of the thick filaments.

In cardiac and skeletal muscle, the smooth endoplasmic reticulum has become specialized to store calcium and to release it rapidly and evenly across the sarcomeres following stimulation. The specialization of the smooth endoplasmic reticulum forms the sarcoplasmic reticulum, a membrane compartment surrounding the sarcomeres (see Fig 11-16). The SR is a specialized adaptation of the ancestral property of the

endoplasmic reticulum to store and release calcium. A high level of Ca^{++} -ATPase (calcium pump) is present in the SR membrane. Ca^{++} is actively concentrated inside the SR, where much of it is bound to protein.

Regularly spaced invaginations of the plasma membrane, the transverse tubules, penetrate the interior of the striated muscle cell, where they associate with two segments of SR to form triads (see Fig 11-16).⁷⁶ Depolarization of the sarcolemma spreads into the TTs to activate voltage-gated L-type calcium channels concentrated in TTs at the triads.³⁵ These channel proteins are physically linked to ryanodine receptors in the SR membrane.⁷⁶ The ryanodine receptors are a special type of calcium channel made up of four identical subunits.

Activation of the ryanodine receptors in response to voltage-initiated changes in the voltage-dependent calcium channels of the TTs allows Ca^{++} to escape from the SR into the sarcoplasm, bathing the sarcomeric contractile apparatus. Reuptake of Ca^{++} , carried out by calcium pumps, in the SR membrane, causes relaxation of the contractile apparatus. Calse-

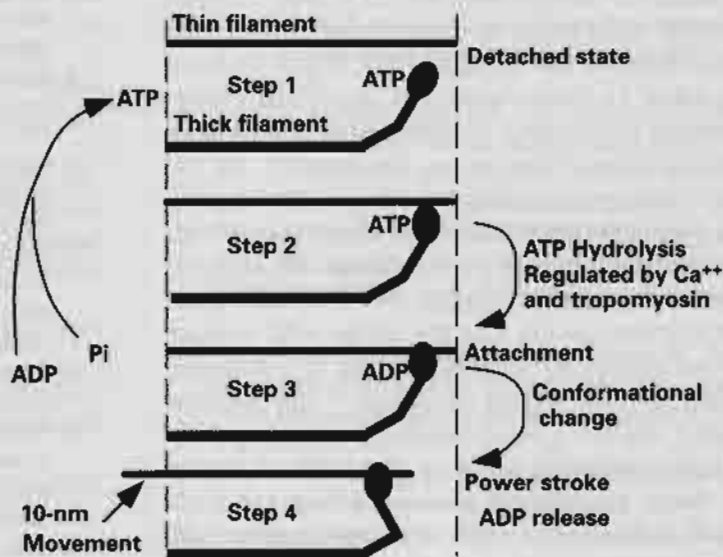


Fig 11-18 Sequential events in adenosine triphosphatase action resulting from the interaction between actin thin filaments and myosin thick filaments during a muscle contraction cycle. (ADP) Adenosine diphosphate; (ATP) adenosine triphosphate; (P_i) inorganic phosphate.

questrin, a calcium-binding protein of the SR, helps to increase the amount of calcium that can be stored in the SR during the relaxation phase.

The activation of the contraction of actin and myosin by Ca^{++} in striated and cardiac muscle is regulated by tropomyosin and the troponins. These proteins control the approximation of the globular enzymatic head of myosin to actin. Tropomyosin, a rod-shaped protein, stabilizes F-actin by binding along its length and joins the troponin complex to the actin filaments. The troponin complex is made up of three subunits; troponins I, C, and T. Subunits I and C are globular. They are attached to the tail-like T subunit, which in turn attaches the entire troponin complex to tropomyosin.

The position of tropomyosin in relation to the actin thin filament controls the interaction of actin and myosin. Troponin I and troponin C act as molecular switches to regulate the position of tropomyosin. Troponin I (the off switch) inhibits the activation of myosin ATPase by keeping tropomyosin in its blocking position along the actin filament. When calcium is released

from the sarcoplasmic reticulum, it binds to troponin C (the on switch), causing troponin I to reposition itself and to release its inhibitory hold on tropomyosin. Tropomyosin is now able to shift its position on the actin filament, allowing a more intimate contact between the myosin globular head group and actin, to activate the ATPase reaction. These events would take place during steps 2 to 3 represented in Fig 11-18. This classic paradigm of the regulatory interactions among troponins, tropomyosin, and actin has been re-examined in the light of new findings and shown to be still valid but oversimplified.⁷⁷

Muscle contraction is caused by the short movements of the myosin head groups, energized by ATP hydrolysis, and triggered by contact with actin thin filaments. The cumulative force of the movement of hundreds of myosin head groups ratchets the actin filaments along the thick filaments toward the M line. This results in a shortening of the I band. In this "sliding" movement, neither thin or thick filaments contract.

The ATPase-powered interaction between myosin and actin is depicted in Fig 11-18. In step 1, myosin

releases its attachment to actin when it binds ATP. Without ATP, thick filaments remain bound to thin filaments, and the muscle soon attains a state of rigor. The release of calcium from the SR in step 2 allows the myosin head group to make closer contact with the actin filament, activating ATPase. This movement is regulated by tropomyosin, as described earlier. The release of inorganic phosphate (step 3) triggers a conformational change in the position of the myosin head group, producing a power stroke (step 4) that moves the actin molecule a distance of 10 nm.

The architecture and the functional efficiency of the sarcomere are truly impressive. It is even more wondrous considering that the sarcomeric components are constantly renewed without any evidence of structural or functional disruption.⁷⁸ The half-lives of α -actin, myosin II, tropomyosin, and the troponins have been measured to be on the order of several days. Newly synthesized proteins diffuse into position and replace older ones while sarcomeres continue to function.

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Chapter 12

Cartilage and Temporomandibular Joint



Development and Structure of Cartilage

Mesenchymal tissues contain stem cells that have the potential to follow divergent pathways of differentiation to form fibrous, osseous, or cartilaginous tissues. Pathway selection is determined by micro-environmental factors, such as oxygen tension, compression, nutrient supply, biomechanical tension, and hormone and growth factor levels. Tensional forces favor the formation of a fibro-osseous (collagen type I) connective tissue, while compressive forces favor the development of a cartilaginous tissue (collagen type II).

Cartilage is a special type of connective tissue designed to survive high compressive forces. Unlike bone, which is resorbed under pressure, cartilage persists and may enlarge by interstitial and appositional growth, even when under compression. Cartilage is also unique in its lack of blood vessels, nerves, and lymphatics. It is ideally designed to act as an articulating surface.

Most articular surfaces are covered by hyaline cartilage, a cartilage high in type II collagen and large proteoglycan (Pg) aggregates. Fibrocartilage has a high content of fibrillar type I collagen in addition to its component of type II and IX collagens. It forms at the insertion of tendons to bone. Elastic cartilage contains an abundance of elastic fibers and is lo-

cated in tissues that require a combination of rigidity and flexibility.

Chondrogenesis

The first sign of cartilage formation involves the condensation of a mass of undifferentiated mesenchymal cells. The formation of a prechondrocytic cell mass requires cell adhesion molecules (N cadherin and neural cell adhesion molecule) plus extracellular matrix (ECM) molecules (fibronectin and tenascin). At the periphery of the cell mass, a perichondral zone is established by spindle-shaped mesenchymal cells that continue to express type I collagen and tenascin C (Fig 12-1).¹

Undifferentiated prechondrocytes within the perichondrium proliferate to give rise to early differentiating chondrocytes. During the conversion to chondrogenic differentiation, the cells must downregulate the production of type I collagen while activating the synthesis of collagen types II, IX, and XI and the large proteoglycan, aggrecan. Recruitment of chondrocytes from the inner layer of the perichondrium permits the cartilage to grow by apposition.

Closer to the middle of the cell mass, in the biosynthetic zone, chondroblasts enlarge as the rough endoplasmic reticulum (RER) and the Golgi apparatus develop (Fig 12-2). Distinct secretory vesicles form in the Golgi apparatus and are secreted in an apparent multipolar mode. Two types of secretory

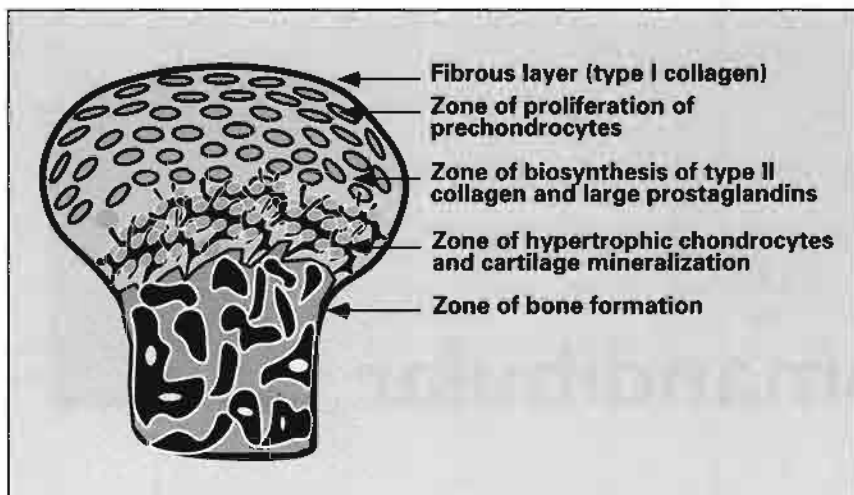


Fig 12-1 Structure of a condylar cartilage. The outer surface is covered by a sparsely populated fibrous layer. Beneath the fibrous layer is a thicker layer containing cartilage stem cells and numerous rows of proliferating transit cells that amplify the pool of differentiating chondrocytes. A middle zone contains numerous plump biosynthetically active cells that secrete the bulk of the cartilage matrix. In the zone of hypertrophic chondrocytes, matrix mineralization begins, and most of the cells eventually undergo programmed cell death just above the zone of bone formation. These zones are shown in Fig 12-8a.

granules are produced in distinct stacks of Golgi cisternae.² Cylindrical granules contain parallel-aligned 300-nm-long "threads" of procollagen. Spherical granules containing dotted filaments contain proteoglycans.² The major secretory products are collagen types II, VI, IX, and XI; a large cartilage-specific Pg (aggrecan); hyaluronate; and several smaller proteoglycans.³

As each chondroblast secretes matrix, it becomes enclosed within a lacunar space, isolated from its neighboring chondrocytes. Interstitial (endochondral) growth of the cartilage occurs as each chondrocyte adds more matrix to its immediate surroundings. Some chondrocytes divide to give rise to a small clone of cells within a single lacunar space. Rapid growth of cartilage occurs primarily as a result of interstitial growth. Perichondral or appositional growth is a slower process.

Chondrocytes are nourished by diffusion of small metabolites from capillaries within perichondral connective tissue and/or bone marrow spaces. Differentiating chondroblasts store energy in the form of glycogen, to be used later in anaerobic metabolic pathways to generate the energy needed for protein and glycosaminoglycan (GAG) synthesis.⁴ In joints, the synovial fluid may serve as a source of nutrients and growth factors for the articular cartilage.

The movement of water and large molecules through the matrix is restricted by collagen type II and the associated large, negatively charged proteoglycan, aggrecan. Large proteins, such as serum proteins, are excluded from the matrix. Unlike osteocytes, chondrocytes do not form canalicular cell processes joined by gap junctions; thus there is no intercellular cytoplasmic pathway for the movement

of small metabolites from cell to cell. Mineralization of the matrix adjacent to older hypertrophic chondrocytes further restricts the diffusion of nutrients.

The final stage of chondrocyte differentiation occurs in the zone of hypertrophic chondrocytes (see Fig 12-1). Depletion of nutrients, a result of restricted diffusion through the cartilaginous gel, may trigger the terminal events in chondrogenesis. Hypertrophic chondrocytes are characterized by dense crenelated nuclei and shrunken profiles of the RER (see Fig 12-2). Hypertrophic chondrocytes secrete collagen type X and express increased levels of alkaline phosphatase activity.³ They also release matrix vesicles (MVs) from the tips of cell processes by a budding process.⁵

Hypertrophic chondrocytes enter a pathway leading to programmed cell death,^{6,7} or, as some recent evidence suggests, some may survive to enter an osteogenic pathway.^{8,9} Calcium released from mitochondria during programmed cell death may contribute to mineralization.⁴ The death of the hypertrophic chondrocytes is associated with vascularization, bone formation, and cartilage resorption. During apoptosis, the cytokine interleukin 1 (IL-1) is produced. It may have a role in triggering osteoclastic (chondroclastic) activity, observed along the base of the mineralized cartilage scaffold.

In recent *in vitro* studies of the growth of cartilaginous explants, it was determined that chondrocytes can undergo asymmetric division, wherein one daughter cell undergoes apoptotic cell death in the hypertrophic zone, while the other daughter cell gives rise to osteogenic cells through further cell division.⁹ Markers of the osteogenic phenotype, such as osteopontin, osteonectin, osteocalcin, bone sialo-

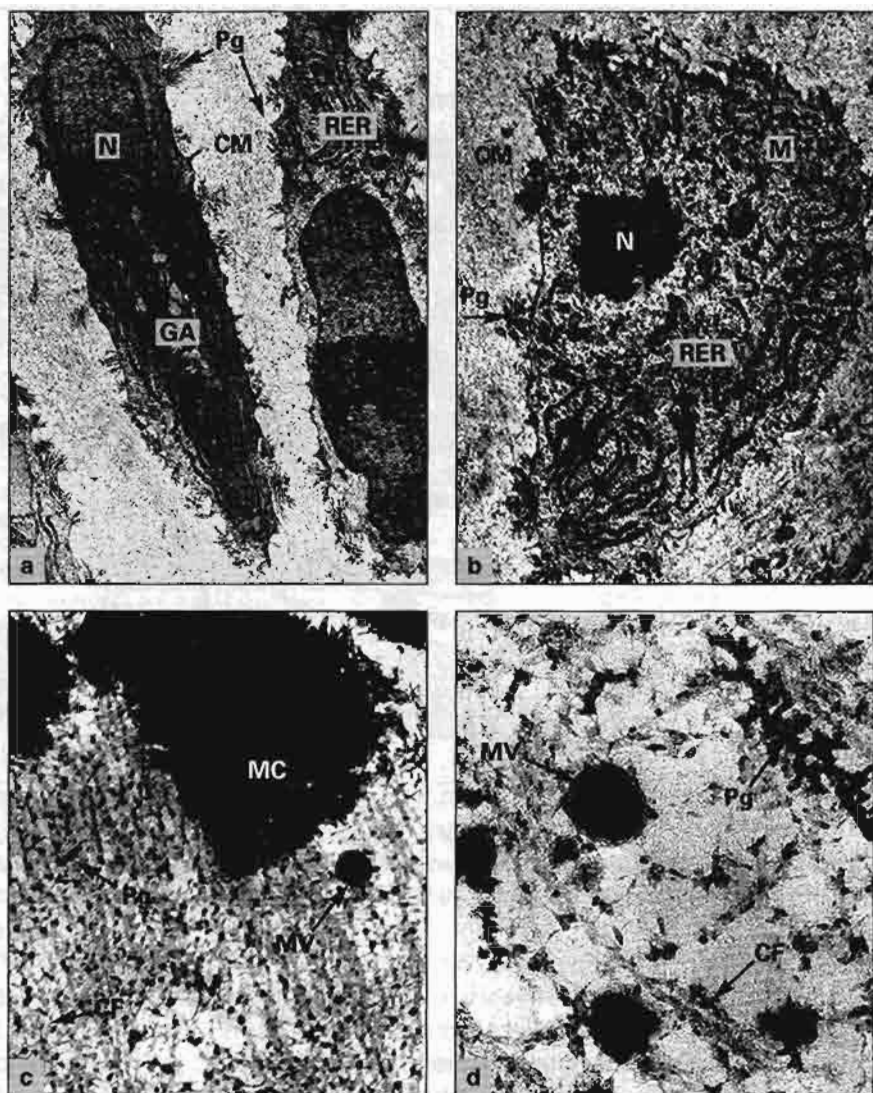
Figs 12-2a to 12-2d Electron micrographs of various stages in cartilage formation.

Fig 12-2a Chondrocytes in the zone of biosynthesis contain an abundance of rough endoplasmic reticulum (RER) and well-developed Golgi apparatuses (GA). Clusters of newly secreted proteoglycan (Pg) are present near the cell membrane (CM). (N) Nucleus. (Original magnification $\times 3,800$.)

Fig 12-2b Chondrocyte in the early stage of hypertrophy demonstrating condensation of the nucleus (N) and the rough endoplasmic reticulum (RER). (CM) Cell membrane; (Pg) proteoglycan; (M) mitochondria. (Original magnification $\times 4,000$.)

Fig 12-2c Cartilage matrix in the zone of mineralization contains matrix vesicles (MV), collagen fibrils (CF), and dispersed proteoglycan (Pg). (MC) Mineralized cartilage. (Original magnification $\times 35,000$.)

Fig 12-2d Higher magnification of the components of the mineralizing cartilage matrix. (CF) Collagen fibril; (MV) matrix vesicle; (Pg) proteoglycan. (Original magnification $\times 72,000$.)



protein, and collagen type I, were detected in viable cells sharing the lacunar space with apoptotic chondrocytes. These observations, as well as some purely morphologic studies, suggest that osteogenic cells can arise by a redifferentiation or transdifferentiation of chondrocytes following division of chondrocytes during endochondral bone formation.^{8,10,11} The microenvironmental factors that control asymmetric division and drive the change to osteogenesis have yet to be identified.

Apoptosis of hypertrophic chondrocytes occurs adjacent to the advancing front of vascularization and chondroclastic activity.⁶ Although it has been suggested that the invading capillary endothelium may generate factors that induce chondrocyte apoptosis, another attractive hypothesis is that the hypertrophic chondrocytes fail to receive appropriate growth fac-

tors or that growth factor receptors are downregulated during the hypertrophic state. In cell types that are highly dependent on the continued presence of hormones and/or growth factors, such as the secretory cells of the prostate and the mammary gland, the abrupt removal of hormone or growth factor stimuli induces apoptosis.¹² In this way, the organism rids itself of superfluous cells once their function is no longer needed. Another possible trigger could be the loss of chondrocyte-to-matrix contact. In summary, apoptosis appears contemporaneously associated with cartilage mineralization, matrix resorption, neovascularization, and osteogenesis.^{6,13}

Matrix mineralization triggers the invasion of the cartilage by endothelium, chondroclasts (osteoclasts), and osteogenic cells. A rich supply of invading capillaries is visible at the junction between the

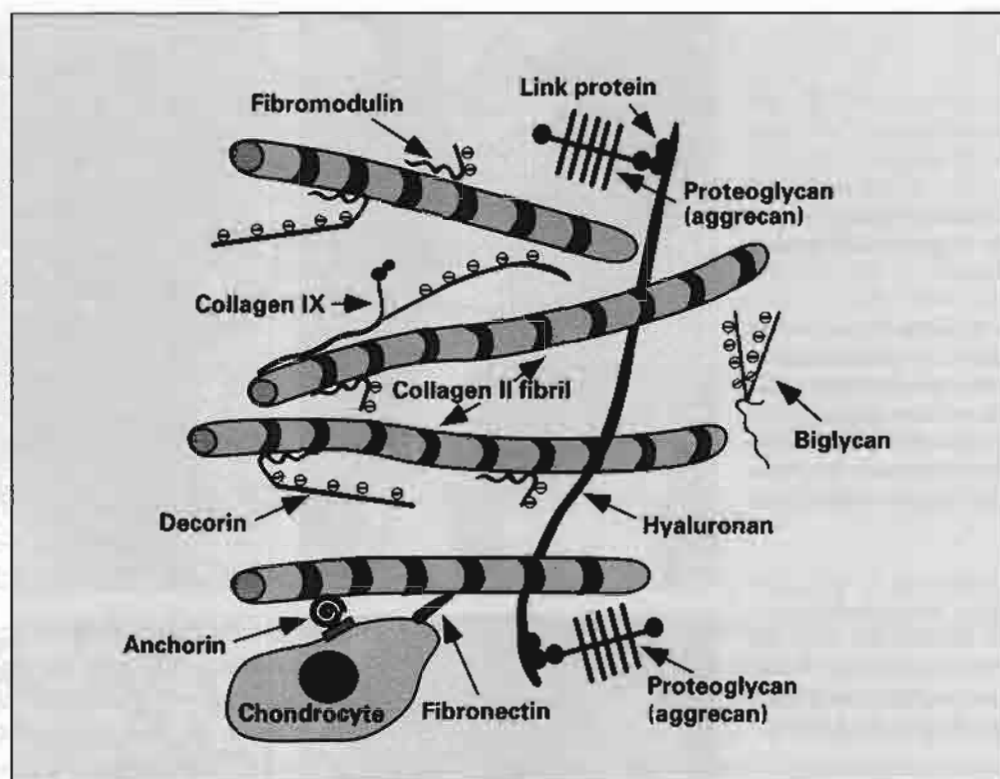


Fig 12-3 Arrangement of the major molecular components of cartilage matrix. The molecular components are greatly magnified in relationship to the chondrocyte. The reader should imagine all empty spaces as being filled by additional aggrecan and other proteoglycans to form a stiff gel in a collagen scaffold. (Adapted from Heinegard and Oldberg²³ with permission.)

hypertrophic chondrocytes and the zone of bone formation.¹⁴ Ultrastructural studies have shown that the capillary endothelial cells enter empty lacunar spaces with leading cytoplasmic processes penetrating the cartilage matrix. During the formation of new capillaries (angiogenesis), endothelial cells secrete proteases that partially degrade and loosen the ECM. A similar process may help to degrade the cartilage matrix. However, with the arrival of numerous monocytes and chondroclasts (osteoclasts) at the base of the hypertrophic zone, there is no shortage of proteolytic enzymes at that site.¹⁴

Despite reports of asymmetric division and possible transdifferentiation of hypertrophic chondrocytes, most evidence supports the thesis that cartilage is replaced by bone following an invasion by bone marrow osteoprogenitor cells along with the growth of new blood vessels. Following the death of the hypertrophic chondrocytes and the vascular invasion of the cartilage, endochondral bone forms when new osteoblasts differentiate after contact with the scaffold of mineralized cartilage^{15,16} (see Fig 12-1).

Hypertrophic chondrocytes are a source of stimulatory factors for vascularization and bone cell differentiation.^{8,17} Matrix metalloproteinase 9 (MMP-9/gelatinase B) was recently discovered to play an essential role in the later stages of the removal of hypertrophic cartilage and in endochondral bone formation.¹⁸ Ablation of the *MMP9* gene leads to a decrease in the vascular invasion of hypertrophic cartilage and delayed apoptosis of hypertrophic chondrocytes. However, the cells that express MMP-9 in the growth plate are not chondrocytes but cells originating from the underlying bone marrow.

It appears that programmed cell death of chondrocytes is coupled to vascular invasion. Although the exact role of MMP-9 in this process is still unclear, it has been suggested that it is involved in the release of vascular endothelial growth factor (VEGF) from cartilage matrix.¹⁸ It has been reported that hypertrophic chondrocytes express VEGF and secrete it into the cartilage matrix.^{17,19} In addition to its angiogenic action, VEGF may coordinate the maturation of chondroclasts and osteogenic cells at the

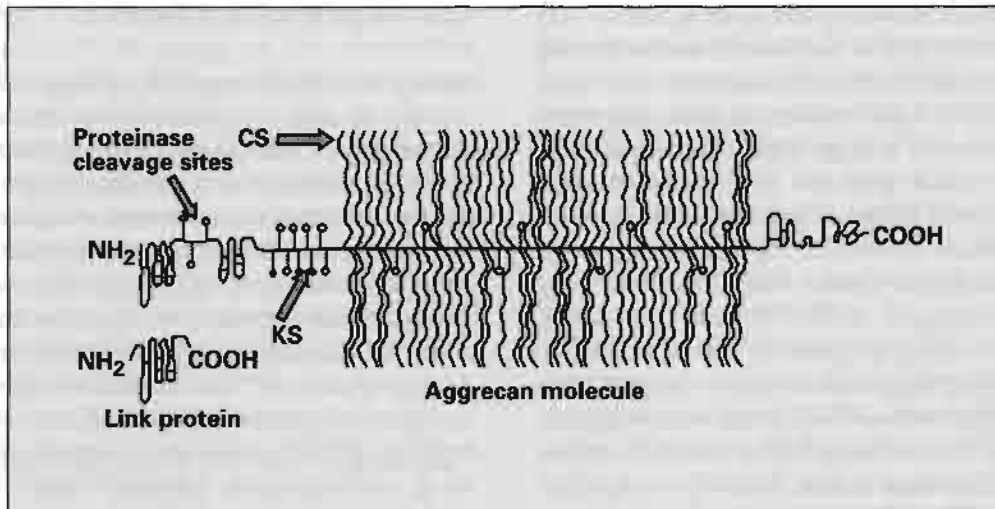


Fig 12-4 Molecular architecture of the aggrecan molecule and the link protein. Note the similarity of the link protein (three looplike segments stabilized by disulfide bonds) to the amino terminal of the aggrecan core protein. (CS) Chondroitin sulfate; (KS) keratan sulfate. (Adapted from Hardingham and Fosang²² with permission.)

vascular invasion front through autocrine-paracrine pathways.¹⁷ Both cell types have receptors for VEGF.^{13,19} When VEGF action is blocked by systemic administration of soluble receptor protein, hypertrophic chondrocytes fail to undergo apoptosis and the removal of cartilage is blocked.¹⁹ Thus, MMP-9 may initiate the replacement of cartilage by bone in the growth plate by releasing VEGF from cartilage matrix.

Cartilage matrix components

The major constituents of the cartilage extracellular matrix are collagen fibrils, comprising types II, VI, IX, X, and XI collagen, and large aggregating proteoglycans, that is, aggrecan (Fig 12-3).²⁰⁻²³

Collagens

Collagen type II represents the major fibrillar component of the cartilage matrix. Several mutations in the collagen II gene have been linked to cartilage abnormalities, such as osteoarthritis and some forms of chondrodysplasia.^{24,25}

Collagen type VI is predominantly found in the superficial zone of proliferation and in the hypertrophic zone of the mandibular condyle.²⁶

Type IX collagen is a fibril-associated collagen. It is constructed of three different α chains, one of which is covalently linked to a GAG chain. It may act as a bridge between the proteoglycans and collagen

fibrils.²⁰ Experiments with genetically engineered mice indicate that abnormalities in the structure of type IX collagen lead to the breakdown of articular cartilage and the development of degenerative joint disease.

Collagen type X is expressed mainly in the hypertrophic zone and is involved in regulating matrix vesicle mineralization. Mutations in type X collagen produce a form of osteochondrodysplasia.²⁷

Collagen type XI, expressed with collagen type II in cartilage, is a member of the fibril-forming collagens that regulates the size of cartilage matrix fibrils.²⁰ Genetic mutation in the collagen XI gene leads to severe defects in cartilage matrix cohesiveness, causing homozygotes to die at birth with widespread skeletal abnormalities.²⁴

Proteoglycans

The aggrecan molecule consists of a core protein to which are attached numerous negatively charged chondroitin sulfate and keratan sulfate GAG side chains^{22,23} (Figs 12-3 and 12-4). The amino terminal domain of the core protein consists of a globular unit that forms a noncovalent bond to hyaluronan. The association between aggrecan and hyaluronan is stabilized by link protein, a molecule whose structure closely mimics that of the amino terminal of aggrecan core protein.^{22,23} A lectinlike globular domain is present at the carboxy end of the core protein.

Each aggrecan molecule has approximately 100 chondroitin sulfate GAGs and fewer keratan sulfate GAGs. Because each chondroitin sulfate chain possesses about 100 negative charges, each aggrecan molecule represents a large fixed negative charge that adsorbs water through a Donnan equilibrium-type osmotic force. In cartilage matrix, hundreds of aggrecan molecules are attached to long, ribbonlike hyaluronan chains that snake their way around the collagen fibrils^{22,23} (see Fig 12-3). Hyaluronan is a long polymer of repeating sugar molecules (*N*-acetylglucosamine and D-glucuronic acid). The polyanionic network of aggrecan-hyaluronan, stabilized by the collagen fibril network, draws water into the cartilage matrix, creating a turgid gel capable of absorbing high compressive forces. In transmission electron micrographs, the proteoglycans are visualized as densely stained granular precipitates associated with the surface of the collagen fibrils.²⁸ In their natural hydrated state (unfixed), the proteoglycans are expanded to fill the spaces between the collagen fibrils.

Other Pgs present in cartilage matrix include biglycan and decorin.²⁹ (see Fig 12-3). Both molecules are much smaller than aggrecan and contain only one or two GAG sidechains each. The role of biglycan has yet to be identified. Decorin binds to collagen types II and I. Another collagen-binding protein contained in cartilage is fibromodulin.²⁹ Both decorin and fibromodulin influence fibrillogenesis and may control the diameter of collagen fibrils.³⁰

Perlecan, a proteoglycan component of the epithelial basal lamina, is also found in cartilage matrix. Homozygous mice with a null mutation in the perlecan gene exhibit chondrodysplasia and a deficient fibrillar collagen network.³¹ This finding suggests that the perlecan proteoglycan protects cartilage matrix from degradation.

Anchoring, a cell surface protein with a binding affinity for collagen type II, acts as an attachment site between chondrocytes and collagen fibrils.³² It may function in a mechanoreceptor process, transmitting information to the cell about the magnitude of compression and/or tension of the ECM.

Cartilage matrix in the resting and differentiating chondrocyte zones contains the antivascularization factors troponin I (the same protein that regulates contraction of skeletal muscle) and chondromodulin 1.^{33,34} The expression of antivascularization factors decreases abruptly in the zone of chondrocyte hypertrophy. Additional proteins, whose functions are still undefined, have been isolated in small amounts from cartilage matrix.

Cartilage mineralization

During the initial stages of cartilage mineralization, crystals develop inside small, membrane-limited matrix vesicles. These vesicles form by budding from the tips of hypertrophic chondrocyte cell processes.^{5,35,36} Matrix vesicles contain alkaline phosphatase and nucleation cores, consisting of phosphatidylserine, calcium, and inorganic phosphate (Pi), which act as seeds for concentrating amorphous calcium phosphate for subsequent precipitation of hydroxyapatite mineral crystals.^{37,38} The limiting membrane contains ion channels that allow inward diffusion of Ca^{2+} and Pi from the ECM.³⁹ Inorganic phosphate is transported via a Na^+ -dependent transport system.³⁹ A specific adenosine triphosphatase provides energy for the Ca^{2+} and Pi transport.⁴⁰

Initially the calcium concentration inside the MV is low (approximating that of the cytoplasm), despite the high driving concentration gradient for calcium entry from the ECM. As the concentration of mineral ions increases inside the MV, mineral crystals grow until they eventually rupture the limiting membrane.³⁵ These crystals can then act as seeds for additional crystals in the ECM through a process of secondary nucleation. Each calcified matrix vesicle forms a growing nodule of hydroxyapatite crystals. These calcified nodules fuse to form trabeculae of calcified cartilage.^{41,42}

Carbonic anhydrase activity in MVs regulates the internal pH to protect nucleation centers from excessive buildup of protons generated during crystal formation.⁴³ A bicarbonate transporter in the MV membrane facilitates entry of HCO_3^- from the extracellular space in exchange for chloride ions. Once inside the MV, the HCO_3^- binds H^+ to generate H_2O and CO_2 in a reverse-hydrolysis reaction catalyzed by carbonic anhydrase.⁴³

A significant step in MV research was made when the regulatory interaction between collagen and MVs was discovered.⁴⁴ The MV transmembrane protein, annexin V, binds to collagen types II and X, thereby activating calcium uptake by the MVs.⁴⁵ Annexin V also participates in forming nucleation cores in the MVs³⁸ and creating calcium channels.⁴⁶

The discovery that MVs contain high concentrations of MMPs that attack proteoglycans, and the observation that proteoglycan degradation accompanies matrix mineralization, has led to the suggestion that MVs are involved in preparing the extracellular matrix for mineralization.⁴⁷

The evidence supporting the matrix vesicle system of cartilage mineralization is strong. Other theo-

ries of cartilage matrix mineralization have been suggested, however. These center on the observation that aggregates of collagen types II and X and associated Pgs act as nucleating centers.⁴⁸

In the growth plate of long bones, mineralization takes place in the longitudinal septal matrix of the hypertrophic zone. The mineralized longitudinal septae serve as a scaffold for osteogenic cells and new bone formation during endochondral bone formation. The unmineralized transverse septae are degraded by MMPs associated with invading macrophages, capillary sprouts, and chondroclasts.

Factors that regulate chondrogenesis

Studies of limb bud development and of the subsequent formation of limb skeletal components have uncovered several families of genes that play crucial roles in the development of cartilage, bone, and joint tissues. Many of the early findings were made in studies of developing chick embryos and later confirmed in genetically modified mouse models. Mutations in the controlling genes were then shown to be the underlying cause of human genetic disorders, including a variety of chondrodysplasias and skeletal hypoplasias.

Wnt gene family

Several members of the *Wnt* family of genes encode secreted glycoproteins that have been shown to be essential to joint initiation (*Wnt-14*) and the regulation of chondrocyte proliferation (*Wnt-4* and *Wnt-5a*).⁴⁹ Other members of the *Wnt* family specify the dorsoventral axis of the developing embryo, as well as the dorsoventral axis of limbs. *Wnt-14* blocks chondrocyte differentiation in the interzone of the future joint, and induces the expression of CD44.⁵⁰ Both are required for tissue cavitation and the creation of a joint space.

Indian hedgehog

The Indian hedgehog (*Ihh*) gene encodes a signaling factor that stimulates chondrocyte proliferation.⁵¹ In the absence of *Ihh*, there is a severe restriction of chondrocyte proliferation, leading to dwarfism.⁵² Homozygous null (*Ihh* -/-) mouse embryos have shortened limbs and hypoplastic mandibles and snouts. Indian hedgehog signaling is also essential for bone formation via upregulation of the expression of *Osf-2/Cbfa1* (a key osteoblast-stimulating factor) and bone morphogenetic protein 3 (BMP-3).

Fibroblast growth factor and its receptors

Fibroblast growth factor (FGF) acts as a powerful mitogen for mesenchymal cells, including prechondrocytes. Signaling by various members of the FGF family through their receptors (FGFR-1, -2, -3, and -4) has been shown to play key roles in limb development through regulation of cell proliferation and differentiation.⁵³ Fibroblast growth factors 10, 8, 2, and 4, signaling through FGFR-1 and FGFR-2, are needed for limb bud development.⁵⁴

Mutations in receptors for FGF lead to achondroplasia and various skeletal abnormalities. Downstream signals from FGFRs lead to decreased activation of *Ihh* and BMP pathways.^{52,55} Mutations in FGFR-2 and FGFR-3 lead to various forms of chondrodysplasia and limb shortening. Hypoplasia of the lower face, resulting from premature closure of sutures, accompanies the limb deformities that are found in FGFR mutations.

Most mutations in the FGFR genes lead to expression of constitutively active receptors.^{56,57} Mutated FGF receptors undergo spontaneous dimerization and transphosphorylation of tyrosine signaling domains in the absence of growth factor binding. Apert syndrome, Crouzon's disease, and Pfeiffer's syndrome are caused by mutations in FGFR-2, while thanatophoric dysplasia, a neonatal lethal form of dwarfism, is the result of certain mutations in FGFR-3.⁵⁶ Patients with these syndromes commonly have severe midfacial hypoplasia and protruding eyes. These patients benefit from remedial plastic surgery and orthodontic realignment of jaw relationships.

Bone morphogenetic proteins

For many years it was recognized that when demineralized bone matrix was implanted into subcutaneous tissue it could induce an endochondral cascade, culminating in the formation of new bone. The active factor was discovered to be a family of BMPs. Bone morphogenetic proteins 4 and 7 promote chondrocyte differentiation and increase Pg synthesis.^{58,59} Joint formation requires both BMPs and BMP antagonists.^{60,61} Absence of BMP antagonists (such as noggin, a BMP-binding factor) leads to excessive chondrogenesis and abnormal joint development.

Insulin-like growth factor 1, growth hormone, and transforming growth factor β

Insulin-like growth factor 1, growth hormone, and transforming growth factor β exert an anabolic effect on chondrocytes⁶²⁻⁶⁴ (Fig. 12-5). Through this ana-

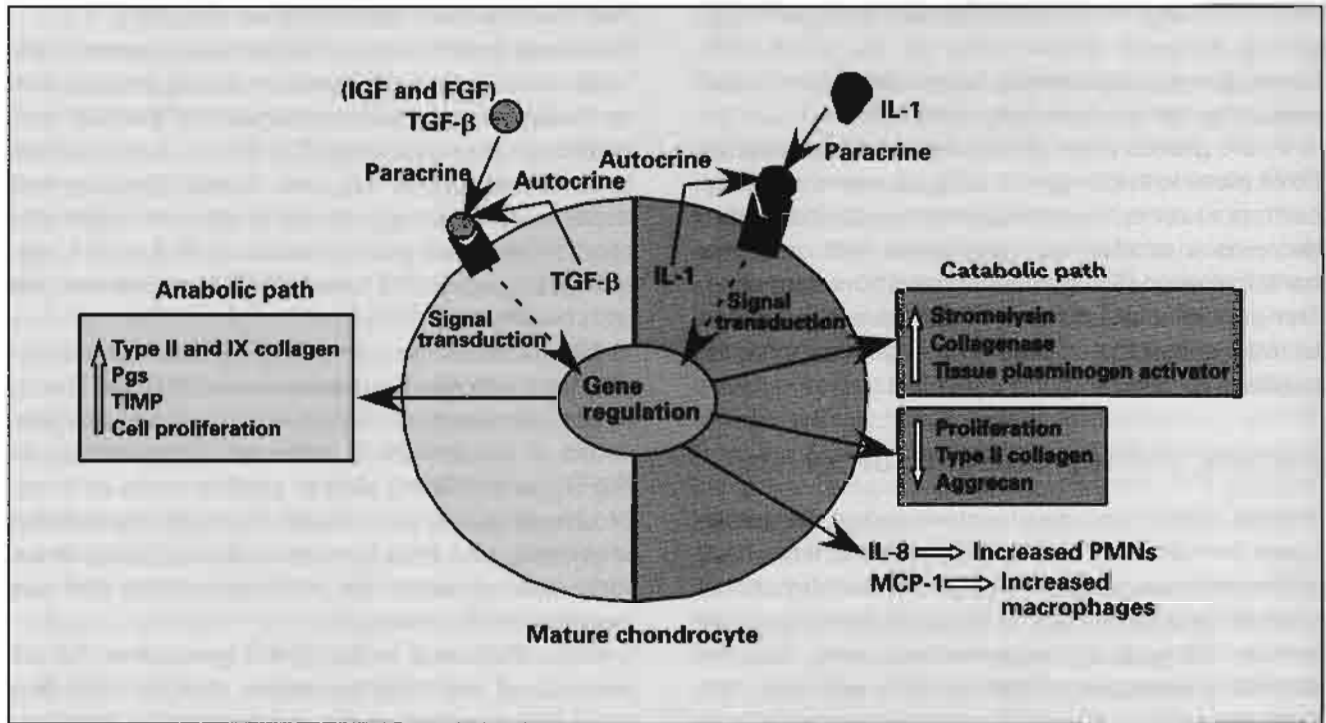


Fig 12-5 Growth factor and cytokine regulation of the anabolic and catabolic states in chondrocytes. Aspects of both pathways may occur simultaneously. In joint disease, the catabolic pathway is amplified in response to stimulatory molecules such as bacterial lipopolysaccharide and exogenous interleukin 1 (IL-1). (FGF) Fibroblast growth factor; (IGF) insulin-like growth factor; (IL-8) interleukin 8; (MCP-1) monocyte chemoattractant protein 1; (Pgs) proteoglycans; (PMNs) polymorphonuclear neutrophils; (TGF- β) transforming growth factor β ; (TIMP) tissue inhibitor of matrix metalloproteinase.

bolic action, transforming growth factor β stimulates collagen and proteoglycan synthesis.⁶⁵ These factors increase the secretion of matrix molecules and stimulate the production of tissue inhibitor of matrix metalloproteinase (see Fig 12-5).⁶²

Retinoic acid

Retinoic acid promotes the maturation of chondrocytes by stimulating the expression of collagen type X, osteopontin, osteonectin, and alkaline phosphatase.⁶⁶ It increases matrix vesicle production by hypertrophic chondrocytes and the rate of mineralization of the matrix.⁶⁶ Retinoic acid also increases proliferation of prechondrocytes.

Mechanical forces

The mechanical forces acting in the microenvironment of differentiating mesenchymal cells are key factors in controlling chondrogenic activity. Static and dynamic compression of cartilage favors the expression of aggrecan, decorin, biglycan, and colla-

gen types I, II, and IX, while tension favors only an increase in decorin.⁶⁷⁻⁷⁰

Compression forces water out of the cartilage matrix, causing a change in ionic concentration as well as a decrease in the pH of the microenvironment surrounding chondrocytes. These effects are a result of the high negative charge that remains bound to the scaffold of collagen, hyaluronate, and aggrecan that surrounds the chondrocytes.

To respond to changing mechanical forces, the chondrogenic cell must possess a mechanism for sensing compression and tension. Many of the hypotheses of mechanoreception in bone cells may apply to chondrocytes. Changes in cell shape that are caused by compression can be communicated to the nucleus via alteration of the cytoskeletal proteins. The flow of water during compression and decompression of the matrix creates shearing forces along the surface of the chondrocytes.

In addition, tension affects the cytoskeleton via matrix-integrin-actin associations at the cell surface. For

example, integrin-mediated release of IL-4 from chondrocytes during mechanical stimulation has been shown to be important in maintaining the chondrocyte phenotype.⁷¹ Interleukin 4 acts in a paracrine-autocrine signaling pathway to promote matrix secretion and to decrease matrix degradation in cartilage.

At this stage of scientific understanding, it is only possible to list potential mechanisms. Much more research is needed before the mechanisms that allow cartilage cells to sense tensional and compressive forces, and the ways in which they respond to this information to maintain homeostasis, are understood.

Cartilage matrix degradation

Although cartilage is a self-renewing tissue with a potential for functioning at high efficiency over a long time, it can be damaged by exposure to unusually high mechanical demands, to trauma, or to inflammatory reactions. Under those conditions, collagen fibers and the Pgs of the matrix are degraded by proteolytic enzymes originating from cells of the articular disk, chondrocytes, synovial cells, or from cells in the inflammatory infiltrate.⁷²⁻⁷⁴ Matrix breakdown products, such as free glycosaminoglycans, aggrecan fragments, and collagen peptides, as well as MMPs are elevated in the synovial fluid of patients with joint diseases.⁷⁵⁻⁷⁷

The collagen network is more resistant to degradation than are the Pgs. Matrilysin (MMP-7) demonstrates high specific activity against cartilage proteoglycans.⁷⁸ Chondrocytes also produce a special metalloproteinase (aggrecanase) that attacks aggrecan.⁷⁹ Proteoglycans are lost preferentially but can be replaced by continued synthesis during a repair phase following inflammation. The loss of collagen is more likely to be an irreversible component of cartilage breakdown.

Interleukin 1 is a potent cytokine that activates the degradative or catabolic pathway in cartilage^{64,80} (see Fig 12-5). The production of IL-1 can occur in chondrocytes, in which case it acts as an autocrine signal, or it can be derived from synovial cells and/or inflammatory cells to act on chondrocytes in a paracrine signaling mode. Through interaction with its receptor, IL-1 triggers signal transduction events involving protein kinase C to promote the secretion of MMPs and tissue plasminogen activator.⁸¹ Stromelysin, matrilysin, collagenase, and cathepsins degrade collagen fibrils and disassociate the aggrecan-hyaluronate complex.^{22,82} Under the influence of IL-1, the synthesis of collagen and Pgs is downregulated, and proliferation of chondrocytes is dimin-

ished.⁸³ The inflammatory reaction is magnified by the ability of IL-1 to stimulate the production of IL-8, which acts as a chemoattractant for neutrophils, and by the production of monocyte chemoattractant protein 1, a chemoattractant for monocytes and macrophages (see Fig 12-5).

Tumor necrosis factor (TNF) is another inflammatory cytokine capable of triggering a potent catabolic reaction in chondrocytes. Alteration in extracellular matrix components may potentiate further degradation through integrin outside-in signaling. For example, chondrocyte integrin binding to fibronectin stimulates the expression of granulocyte-macrophage colony-stimulating factor and IL-6, which stimulate the differentiation of chondroclasts.⁸⁴

Biologic molecules that interfere with cytokine ligand-receptor interactions have recently been identified. These substances offer the possibility that they may be used clinically to minimize tissue damage during inflammatory joint diseases. These molecules appear to represent mechanisms that cells have developed to turn off or modulate the negative catabolic effects of cytokines.⁸⁵ For example, interleukin 1 receptor antagonist can occupy the IL-1 receptor site and thereby block the effect of IL-1. Along similar lines, the action of TNF may be modulated by the presence of soluble TNF receptors. They bind TNF in the extracellular fluid, thereby reducing the amount of TNF available to the cellular receptor. The potential beneficial effects of IL-1 receptor antagonist and soluble TNF receptor in modulating the catabolic response of chondrocytes *in vivo* have yet to be proven.

Components of the Temporomandibular Joint

The temporomandibular joint (TMJ) consists of the condylar head of the mandible articulating with the glenoid or temporomandibular fossa of the temporal bone (Fig 12-6).⁸⁶ The articulating surface of the temporal bone is convex anteriorly at the tubercle and concave posteriorly at the fossa. The articulating surface of the condyle and the opposing surfaces of the temporomandibular fossa, including the articular tubercle, are covered by fibrocartilage. Chondrocytes in the posterior aspect of the mandibular condyle, a region exposed to greater tensional forces than compression, express collagen types I and II.⁸⁷ Experimental studies indicate that mechanical forces acting on the mandibular condyle play a significant role in the proliferation, differentiation, and rate of maturation of chondrocytes.^{70,88,89}

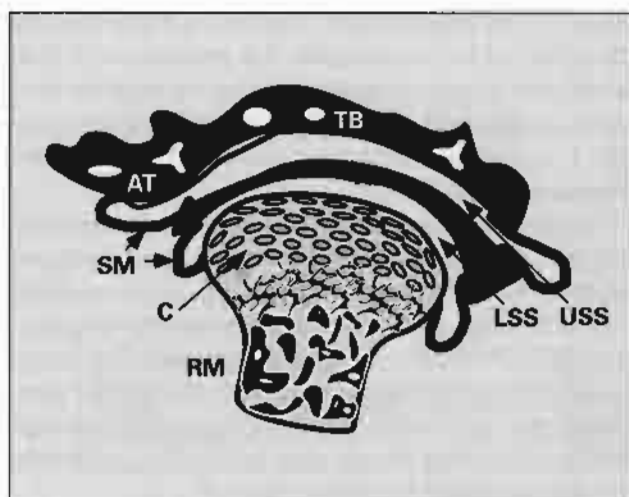


Fig 12-6 Components of the temporomandibular joint. (AD) Articular disk; (AT) articular tubercle; (C) condylar cartilage; (LSS) lower synovial space; (RM) ramus of the mandible; (SM) synovial membrane; (TB) temporal bone; (USS) upper synovial space.

Articular capsule and disk

The joint is enclosed by an articular (joint) capsule of connective tissue that is attached to the fossa, to the tubercle of the temporal bone, and to the neck of the condyle. A thick plate of dense, fibrous connective tissue, the articular disk, is located between the condyle and the articulating surface of the temporal bone. The disk is anchored around its lateral borders to the articular capsule and anteriorly to the external pterygoid muscle by a fibroelastic connective tissue.⁹⁰ The masseter and temporalis muscles also have fibrous connections to the anterior and lateral edges of the articular disk. Upper and lower spaces that are filled with synovial fluid separate the articular disk from the articulating surfaces. As the jaw is opened, the condyle rotates against the disk, and in the final phase of opening it translates anteriorly along the fossa and the inclined surface of the tubercle, gliding on the intervening articular disk.

The articular disk contains spindle-shaped cells that combine the properties of chondrocytes and fibroblasts. These cells synthesize collagen types I, II, and IX and cartilage-specific proteoglycans.⁹¹ They also express the MMPs and tissue inhibitor of matrix metalloproteinases needed for turnover and repair of the fibrocartilaginous ECM. The surface of the articular disk is covered by a cell-free, proteoglycan-rich lamina.⁹² This lamina is supported by

the underlying dense, fibrocartilaginous connective tissue.

The joint capsule and the articular disk are innervated by small, unmyelinated sensory nerve fibers of the A-delta and C types (Fig 12-7).⁹³ These fibers originate in the trigeminal ganglion and are considered to conduct nociceptive information. Many of these nociceptors contain substance P and calcitonin gene-related peptide.^{94,95} The release of substance P from activated and/or injured sensory nerve terminals can produce a neurogenic inflammatory response with vasodilation and serum extravasation. Encapsulated receptors of the Ruffini type are sparsely distributed in deeper connective tissue of the capsule and ligaments.

Condyle

During the development of the condyle, growth occurs by perichondral apposition and by endochondral (interstitial) expansion.¹⁵ Perichondral growth takes place as progenitor cells proliferate to form a prechondroblastic layer. Interstitial growth, resulting from the enlargement of chondrocytes and the secretion of perilacunar matrix, is principally responsible for rapid condylar growth and is especially prominent early in the development of the mandible.^{15,96} During development, the height of the ramus increases as a result of combined condylar growth and associated endochondral bone formation.^{71,97}

Newly differentiated chondroblasts are randomly dispersed in the zone of matrix biosynthesis (Figs 12-8a and 12-8b). After a period of matrix deposition, the chondrocytes undergo hypertrophy and most die as the matrix becomes mineralized. Histologic studies of mandibular condylar growth suggest that some hypertrophic chondrocytes survive and are released into the adjacent marrow compartment. It remains to be established if they transdifferentiate into osteogenic cells.

Empty lacunar spaces and discontinuity of the mineralized intercellular partitions create spaces for vascular invasion, chondroclastic activity, and osteogenesis. Bone forms over the naked ends of the mineralized cartilage strands, thereby fusing the condylar cartilage to the osseous mass of the ramus. The sequence of differentiation is similar to that of endochondral bone formation at the growth plate.¹⁵ In the growth of the mandibular condyle, however, there is no growth plate and no orderly formation of columns of chondrocytes, such as are needed for the axial growth at the epiphyseal plate of long bones. In the mandible, condylar expansion takes place in a multi-

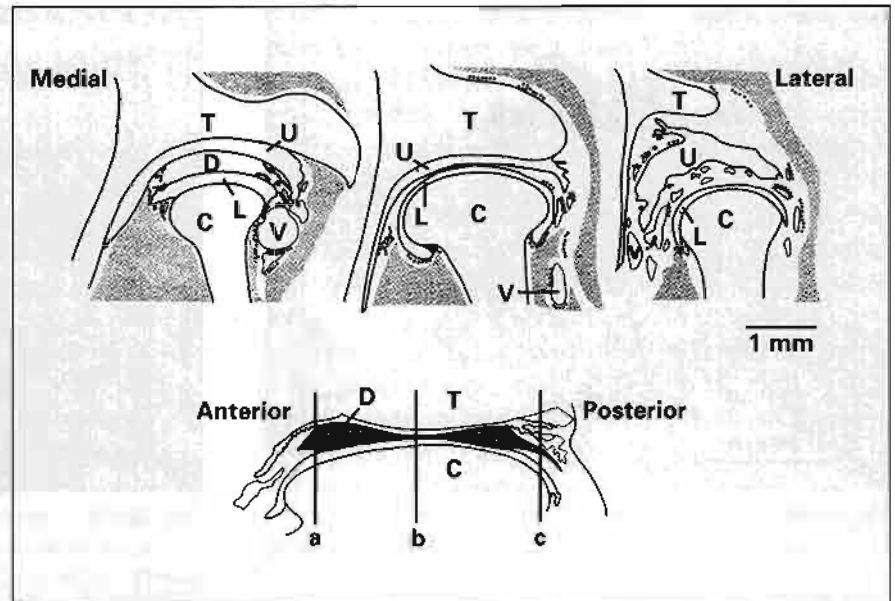


Fig 12-7 Camera lucida drawings of representative frontal sections taken in an anteroposterior axis of the rat temporomandibular joint. (a, b, c) indicate the positions of the frontal sections. Black dots represent location of nerve fibers or fiber groups. (T) Temporal bone; (U) upper space; (D) disk; (L) lower space; (C) condyle; (V) blood vessel. (Reprinted from Kido et al⁹³ with permission.)

Figs 12-8a and 12-8b Histologic sections of rat temporomandibular joint stained with hematoxylin and eosin.

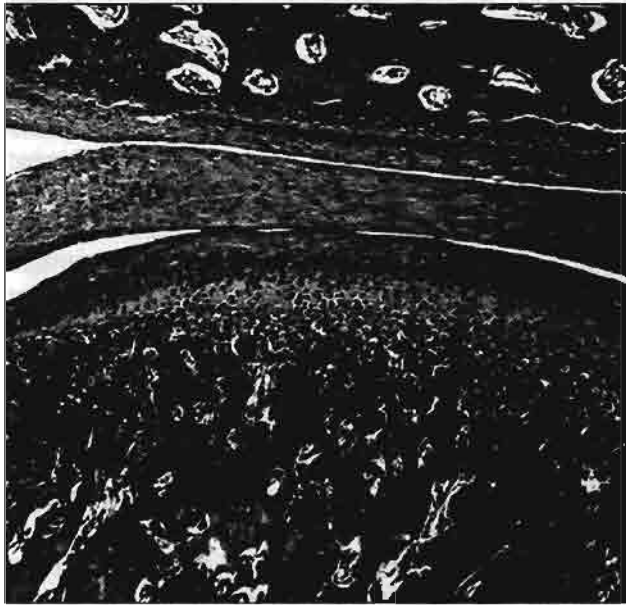


Fig 12-8a Low-power view of articular cartilage, the articular disk (AD), and the surface of the temporal bone (TB) and temporomandibular fossa (BF). (1) Fibrous layer; (2) zone of proliferation; (3) zone of biosynthesis; (4) hypertrophic zone. (Original magnification $\times 120$.)

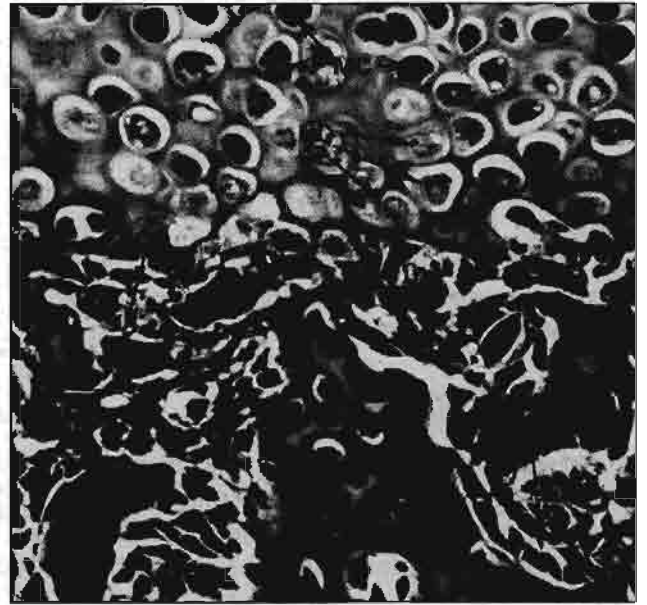


Fig 12-8b Higher magnification of the junction between the hypertrophic chondrocytes (HC) and the bone. (BT) Bone trabeculae; (MC) mature chondrocytes; (OB) osteoblasts; (OC) osteoclasts (chondroclasts). (Original magnification $\times 400$.)

axial mode to adapt to growth in the base of the cranium as well as to accommodate elongation of the mandibular ramus.

Unlike the articulating surfaces in most joints of the body, the mandibular condyle is not covered by

naked hyaline cartilage. Instead, its articulating cartilage surface is covered by a thin layer of poorly vascularized dense connective tissue with few fibroblasts (see Figs 12-1 and 12-8a). Collagen type I is the major constituent found in this outer layer.

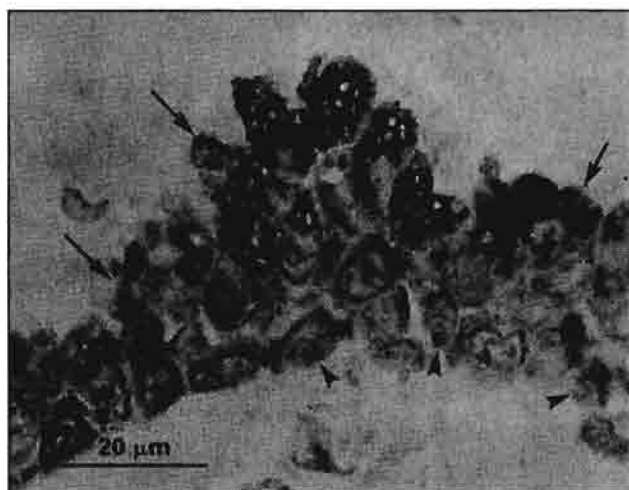


Fig 12-9a Immunolocalization of cathepsin D in synovial membrane. Type A cells (arrows) contain many cathepsin D-positive bodies, while the B cells (arrowheads) are only weakly stained. (Adapted from Kiyoshima et al⁹⁹ with permission.)



Fig 12-9b Control section stained without the primary antibody to cathepsin D. (arrows) Type A cells; (arrowheads) type B cells; (C) capillary. (Adapted from Kiyoshima et al⁹⁹ with permission.)

However, during its developmental phase, the growing condyle is covered by a perichondrium and its bulk is made up of typical hyaline cartilage (see Figs 12-8a and 12-8b). A similar fibrous layer covers the surface of the temporomandibular fossa and articular tubercle. The inner region of this fibrous layer retains progenitor cells that give rise to prechondrocytes.

Synovial tissue

A synovial membrane covers the inner face of the joint capsule. The synovial membrane does not cover the articulating surfaces and the corresponding parts of the articulating disk (see Fig 12-6). The synovial membrane is neither an epithelial lining nor an actual membrane; rather it is composed entirely of connective tissue rich in collagen V. The synovial membrane is well vascularized with numerous fenestrated capillaries.

The synovial membrane is populated by deep stromal cells and two types of lining cells: macrophage-like cells (type A cells) and fibroblast-like cells (type B cells).^{98,99} The type A lining cell rests on a bed of collagenous fibers and protrudes apically into the fluid-filled synovial space. This cell contributes to the formation of viscous synovial fluid and removes and degrades particulate material taken up from the synovial fluid.

Type A cells contain large amounts of cathepsins B, D, and L (Figs 12-9a and 12-9b).^{98,99} Under normal conditions, these enzymes are retained inside the cell and participate in lysosomal degradation of material taken into the cell by endocytosis. When these cells become activated, however, the enzymes may escape from the cell and attack the connective tissue and cartilage matrix.¹⁰⁰

It has also been suggested that the type A cell may be involved in the initial phase of the immune response of rheumatoid arthritis, possibly through antigen processing and presentation.¹⁰¹ Histopathologic studies indicate that there is substantial variation in the morphology of the synovial lining of the normal TMJ, and that synovial inflammation of the TMJ tends to be less severe than that arising in other joints.¹⁰²

In inflammatory joint disease, macrophages and neutrophils enter synovial tissues. They contribute more cathepsins to the joint fluid and cause detrimental changes in the cartilage matrix. In rheumatoid arthritis, inflammatory cells infiltrate the synovial membrane. When this occurs, the stromal and lining cells secrete MMPs (collagenase and stromelysin) into the stromal connective tissue and into the synovial fluid. The MMPs can attack the cartilage matrix as well as the fibrous connective tissue. Collagenase degrades collagen types I, II, and III, and stromelysin is capable of degrading the core protein of the Pgs.⁸²

Clinical Correlation: Pathoses of the Temporomandibular Joint

Pain and dysfunction of the jaw-opening and jaw-closing mechanisms can be caused by a variety of conditions. The causation and clinical management of these temporomandibular disorders are subjects of considerable controversy.¹⁰³ In many cases, the pain is of muscular origin and there is no structural abnormality in the joint. Less commonly, the problem can be traced to structural derangements and/or pathoses in the articulating tissues of the joint.

Abnormalities of the TMJ may develop as part of a generalized systemic disease or they may arise as a result of local causes. The most common local abnormality affecting the joint is a disturbance of the normal anatomic relationship between the condyle and the articular disk.^{104,105} In the healthy joint, the articular disk is tightly adapted to the head of the condyle by medial, lateral, and posterior ligaments. During opening of the mouth, rotational movement occurs between the condyle and the inferior surface of the articular disk. In the last phase of opening, the disk-condylar complex translates anteriorly along the surface of the articular eminence of the mandibular fossa.

Anterior disk displacement, a relatively common joint disorder, may develop after rupture of posterior ligaments or from inflammatory changes in the connective tissues of the posterior part of the articular disk, which weaken the attachment of the articular disk to posterior ligaments. Anterior disk displacement is manifested by pain on function, joint noises of a clicking or popping type, and deviation of the mandible toward the affected side during opening. Histopathologic changes in the articular disk include neovascularization, cell clustering, and fibrillation.¹⁰⁶ Hemorrhage and fibrosis of the condyle and papillomatous hyperplasia of the synovial membrane are also evident following anterior displacement.¹⁰⁷⁻¹⁰⁹

Systemic diseases that affect many joints of the body, including the TMJ, include osteoarthritis. This condition is caused by excessive stress on joints over time, leading to degeneration of the cartilage matrix. Inflammation with hyperplasia and subsequent fibrosis of the synovium may be present as a complicating factor.¹¹⁰ Genetic disorders of the molecular components of the matrix may predispose individuals to osteoarthritis.

Other systemic inflammatory attacks on joint tissue occur in rheumatoid arthritis. The etiology of rheumatoid arthritis remains unresolved. Autoim-

mune, genetic, and bacterial antigen-based hypotheses have been proposed.^{20,111,112}

In each of the aforementioned disorders, the catabolic activities of the chondrocytes are activated, thereby compromising the ability of the chondrocytes to maintain the normal balance of Pgs and collagen matrix components, despite the fact that the synthesis of matrix may be elevated.¹¹³ The loss of Pgs and collagen fibers may reach an irreversible state. When the ability of the cartilage to resist compression declines, it converts to a fibrotic tissue. Increased release of MMPs and cathepsins, as well as IL-1, by synovial and inflammatory cells accelerates the destructive process.^{114,115}

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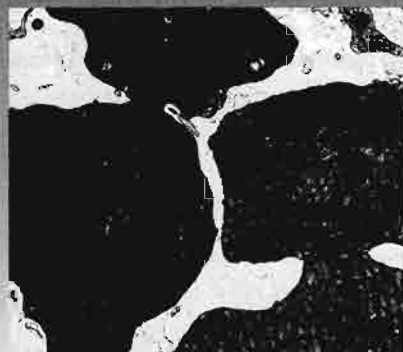
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Chapter 13

Immune System



During the course of evolution, a complex immune system arose for the recognition of non-self from self at the molecular level. Whether it is an invading bacterium, a virus, or a host cell that has acquired new characteristics that make it no longer responsive to normal controls, the offender must be identified and destroyed.

The oldest form of defense is provided by the innate immune system. This system is based on the evolution of host cell (and soluble) pattern recognition receptors and natural antibodies that bind specific classes of complex carbohydrate molecules expressed on microbial surfaces.¹ Pattern recognition proteins are preexistent, waiting for some future encounter with a known foreign invader. Pattern recognition molecules include mannose-binding lectin and Toll-like receptors that act as receptors for surface components of Gram-positive and Gram-negative bacteria, respectively. Receptor-ligand interactions on host defense cells, such as monocytes, macrophages, and neutrophils, activate several innate systems for killing the invading organisms. The complement system is a key component of the innate immune system. Additional discussion of innate immunity is contained in chapter 14.

This chapter will focus on the adaptive immune system, involving highly specific recognition of foreign proteins and/or polysaccharides by surveillance cells and the subsequent activation of antigen-specific T and B lymphocytes. Unlike pattern recognition

molecules of the innate immune system, the defending proteins (antibodies) of the adaptive immune system are extremely specific and manufactured on order. They are the product of a more sophisticated system, one that enables the host to develop a defense against a totally new foreign protein.

The tasks of surveillance and defense are enormous, requiring a division of labor among several cell types: special cells designed to identify foreign elements and effector cells and molecules specialized to destroy the foreign invaders²⁻⁴ (Fig 13-1). Immune system regulation, especially that of the mucosa-associated lymphoid tissue and duct-associated lymphoid tissues of the salivary glands, is of paramount importance to maintenance of the health of oral tissues. Key cells of the immune system regulate, and in turn are regulated by, resident epithelium and connective tissue cells through a host of soluble mediators (cytokines, chemokines, and interleukins) as well as through direct cell-to-cell interactions.^{5,6} Knowledge of this extremely complex interactive system will lead to better treatment protocols for managing pathologic oral conditions.

The uniqueness of each individual is expressed primarily in a spectrum of cell surface glycoproteins. With the exception of identical twins, each human being carries a unique molecular identity determined by slight differences in the sequence of amino acids and carbohydrate residues in many of its molecular components, especially at the cell surface. To iden-

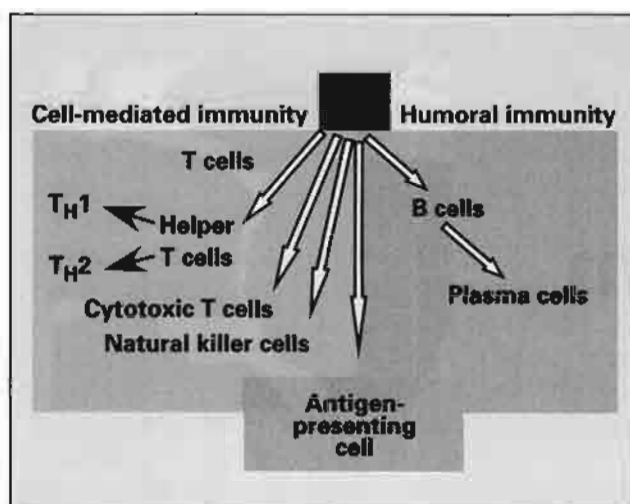


Fig 13-1 Components of the immune system. The immune system is made of T cells, natural killer cells, antigen-presenting cells, and B cells. These populations are renewed from bone marrow stem cells. T cells are involved in cell-mediated responses, while B cells provide humoral immunity.

tify foreign molecular configurations, special cells (lymphocytes) developed the ability to produce antigen receptors (antibodies) through a process of extensive rearrangement of immunoglobulin (Ig) genes. The genes controlling the transcription of the antigen receptor-antibody molecules could be thought of as representing decks of cards; the sequence of the cards dealt would provide the message for the amino acid sequence of the antigen receptor-antibody molecule.

Each antigen receptor is composed of nonvariable domains and a variable antigen recognition site. As each new lymphocyte differentiates, it shuffles its deck of cards to come up with a new and random sequence for the variable segment of its antigen receptor. Because millions of lymphocytes are produced each day, millions of antigen receptor molecules with slightly different antigen recognition sites are constantly entering the surveillance side of the immune defense system. It has been estimated that the human antigen recognition repertoire includes up to 10^{15} different molecules.

Each lymphocyte displays antigen receptors of single-antigen specificity on its plasma membrane. They bind matching antigens, be they on the surface of an invading organism or in the form of soluble antigens in the extracellular fluid. The binding of the antigen to its specific antigen receptor initiates a complex set of cytoplasmic and nuclear reactions in the responding lymphocyte, designed to amplify the de-

fensive reaction by producing more lymphocytes with the same antigen specificity through clonal expansion.

The system is made more efficient by the assistance of antigen-presenting cells (APCs). The APC functions as a collecting and processing unit that displays the foreign antigen (actually a peptide segment of the antigen) on its plasma membrane so that it may interact with the appropriate antigen receptor on a lymphocyte. Antigen-presenting cells as well as other cell types use major histocompatibility complex (MHC) molecules to bind and display antigenic peptides on their cell membrane. In humans, the MHC molecules are also known as human leukocyte antigens (HLAs).⁷ The APC also activates cytoplasmic signals needed for further development of the responding lymphocyte. Many cell types in the body perform APC functions, but dendritic cells are specifically designed for that function.

This chapter will briefly review the components of the immune system, emphasizing lymphocytes. For a review of the immune system covering recent advances, the series by Delves and Roitt^{2,3} should be consulted. There are two major classes of lymphocytes: thymus-derived lymphocytes, or T lymphocytes (T cells), and bone marrow-derived lymphocytes, or B lymphocytes (B cells) (see Fig 13-1). The T cells are involved in the cell-mediated immune response, while the B cells are responsible for the humoral immune response. The T cells interact with other cells over short distances, performing either a stimulating (helper T [T_H] cells) or killing (cytotoxic T cells) function. The humoral system involves secretion of large amounts of antibodies into the extracellular fluid by plasma cells. The B lymphocytes bind intact antigens on cell membrane antibody, while the T cells bind antigenic peptides processed and displayed on MHC molecules on the surface of target cells and antigen-presenting cells.

Initiation of Immune Response

The immune response is initiated by molecules (immunogens) recognized to be foreign by the cellular components of the immune system. The term *antigen* is commonly used as a synonym for *immunogen*. The two terms are not exactly equal, because not all antigens are capable of initiating an immune response, although they both bind to antibodies. Despite this difference, in this discussion the term *antigen* will be used to describe a molecule that generates an immune reaction.

Antigens are typically complex proteins and/or polysaccharides. The "foreignness" of an antigen is subdivided into units of variable size, called *antigenic determinants* or *epitopes*. Epitopes are short, linear chains of amino acids on proteins or monosaccharides on polysaccharide antigens. Usually polysaccharides must be bound to a carrier protein to become immunogenic. Epitopes can also be formed nonlinearly by protein folding; in that case, they are known as *conformational determinants*.

Large proteins have many epitopes; each epitope, recognized by its shape and charge as a foreign entity, engages a separate and specific antibody and/or T-cell receptor. A single immunogen may contain epitopes that activate both T cells (cell-mediated immune response) and B cells (humoral immune response). Not all antigens act in a similar manner when activating an immune response. Some antigens are T-cell dependent. Such antigens require T_H-cell-B-cell interactions to generate an antibody response. The T-cell-dependent antigens are mostly proteins that require intracellular processing by antigen-presenting cells. The T-cell-independent antigens, mostly polysaccharide in nature, act directly on B cells.

Still other antigens (superantigens) activate large numbers of T and B cells in a polyclonal non-antigen-specific interaction. These superantigens activate T cells by binding simultaneously to components of the T-cell receptor (TCR) and the MHC-II molecule that lie outside the antigen-binding groove. Superantigens need not be processed and presented by APCs. Because the response to superantigens is polyclonal, great quantities of cytokines are generated quickly, causing serious systemic consequences. Many bacterial toxins act as superantigens.

Depending on its nature, concentration, and mode of administration, the immunogen will lead to one or more of the following responses: antibody synthesis, cell-mediated immunity, or tolerance.

Development of T Lymphocytes

During embryonic development, stem cells of the immune system are located in the yolk sac, fetal liver, and bone marrow (the primary immune tissues). Stem cells leave the primary immune tissues to undergo further differentiation in the thymus, lymph nodes, spleen, and mucosa-associated lymphoid tissue (MALT), the secondary lymphoid tissues. The adult bone marrow continues to be a source of stem cells throughout the lifetime of the individual.

The thymus gland is derived from a downgrowth of epithelium from the third pharyngeal pouch. It is enclosed in a connective tissue capsule and is partitioned into lobular subdivisions by connective tissue septae. The outer portion of each lobule, the cortex, contains parenchymal epithelial cells and numerous proliferating T cells. The central region of the lobule, the medulla, contains more mature T cells, epithelial cells, numerous macrophages, and dendritic cells as well as blood vessels. The epithelial cells undergo constant turnover, being replenished in the cortex and removed in the medulla. In the medulla, older epithelial cells aggregate into nodules, Hassall's corpuscles, where they degenerate and are phagocytosed and degraded by neutrophils and macrophages.

Epithelial cells of the cortex form a nourishing reticulum whose spaces provide the location for the differentiation of T (thymus) lymphocytes. The epithelial cells have a dual function. They secrete growth substances that promote lymphocyte proliferation, such as thymopoietin and interleukin 2 (IL-2), and they expose lymphocytes to self-antigen and antigen-presenting molecules of the major histocompatibility complex.⁸ The epithelium and its associated basal lamina provide a barrier to the general extracellular fluid and to foreign antigens that may be contained in that fluid. However, the extracellular spaces of the medulla, and the macrophages and dendritic cells located therein, are exposed to blood-borne antigens.

Stem cells of the T-lymphocyte line develop in the bone marrow and migrate via the bloodstream to the cortex of the thymus. In a protected environment provided by the epithelial cells of the thymus, the T cells (lymphoblasts) undergo repeated cell division and rearrangement of the genes that code the TCR antigen recognition site. In T cells, the antigen recognition molecule is not an antibody (immunoglobulin molecule), but a related protein complex, the T-cell receptor.

Like other cell types, T cells express a variety of cell surface glycoproteins during their maturation. These molecules have a variety of functions, serving as receptors, adhesion factors, and enzymes. Because they are expressed at different stages in the lifetime of a specific cell type, they provide useful markers for identifying cells and assessing their level of differentiation.⁹ A standardized nomenclature has been adopted to classify these marker proteins. The cluster of differentiation prefix (CD) identifies more than 100 specific cell membrane molecules that are expressed on white blood cells (many of these same molecules are also found on other cell types).

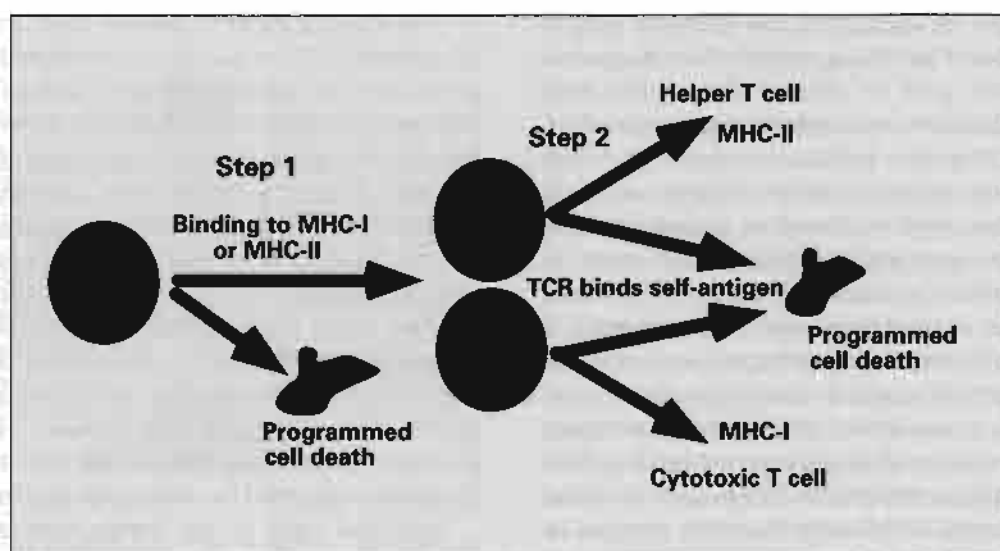


Fig 13-2 Deletion of self-reacting T cells in the thymus. In step 1, $CD4^+CD8^+$ cells that bind to major histocompatibility complex type I (MHC-I) or type II (MHC-II) molecules via T-cell receptor (TCR) are selected for survival. T cells that fail to bind to MHC molecules undergo programmed cell death. During step 2, those T cells that have strong binding affinity to self-antigen via the TCR-MHC axis undergo apoptosis. Helper T cells ($CD4^+CD8^-$) survive to interact with MHC-II-bearing antigen-presenting cells and B cells. Cytotoxic T cells ($CD4^-CD8^+$) survive for interaction with all cells bearing MHC-I molecules.

Two cell surface proteins, CD4 and CD8, define the two major subdivisions of the T-cell lineage. When just entering the cortex of the thymus, T-cell lymphoblasts are $CD4^-$ and $CD8^-$ ("double negatives"), meaning that they are not expressing the CD4 and CD8 glycoproteins.⁸ During maturation, the T cells express TCRs and become $CD4^+$ or $CD8^+$. The importance of the CD4 and CD8 molecules in T-cell function will be explained in a later section. For the time being, it should be noted that T cells expressing CD4 become helper T cells involved in immune regulation, while those cells expressing CD8 become cytolytic T cells, capable of destroying a variety of cells including tumor cells and virus-infected cells in a process requiring cell-to-cell contact (Figs 13-1 and 13-2). The $CD4^+$ T cells interact with antigenic peptide presented on MHC class II molecules, while the $CD8^+$ T cells interact with antigenic peptides presented on MHC class I and CD1 molecules.^{10,11}

During the proliferation and differentiation process, many T cells express TCRs with antigen recognition sites that bind self-antigens. Those T cells that express TCRs that have strong affinity for self-antigens must be selected for destruction before they leave the thymus gland.¹² Only TCRs with a "physiologic" level of binding strength to self-antigens are permit-

ted to survive. This filtering process involves the interaction of the newly differentiating T cells with thymocytes and dendritic cells.¹³⁻¹⁵

Self-reacting T cells are eliminated in the thymus by a two-step process of clonal deletion (see Fig 13-2). Step 1 involves the positive selection and survival of those T cells that bind, via CD8 or CD4 and the TCR, to the polymorphic parts (external to the antigenic peptide-binding site) of MHC-I or MHC-II molecules expressed on epithelial cells in the cortical region of the thymus.^{7,8} Antigenic peptides bound in the antigen-receptor domain of the TCR are not involved in this recognition step. All T cells that fail to bind to self-MHC molecules undergo programmed cell death in the thymus (negative selection).

In step 2, the survivors of step 1 must be screened to identify those T cells that express TCRs that have strong affinity to self-antigens. Self-antigens are processed and displayed in the antigen-binding pocket of the MHC-I and MHC-II molecules on APCs (macrophages and dendritic cells) in the corticomedullary zone of the thymus. The T cells that bind to self-antigens with high affinity on the APCs are eliminated by positive selection and apoptosis.^{7,16} Only T cells that bind with low affinity to self-antigens are allowed to survive. This process is known as cen-

tral tolerance. It is estimated that 98% of all T cells entering the thymus are selected to undergo programmed cell death.⁸

Recent studies of the role of the thymus in the elimination of self-reacting T cells have shown that the epithelial cells located in the medullary region express and display antigenic peptide repertoires that mimic those found in distant tissues, such as the liver and the pancreas.¹⁵ These findings suggest that central tolerance encompasses a larger range of self-antigens, including "peripheral" antigens, than was initially thought possible. The role of the thymic dendritic cells in screening T cells reactive to soluble extracellular proteins has also been extended to many proteins previously thought to be excluded from the thymic environment.¹⁵

Because not all self-antigens are encountered in the thymus, there must be an additional mechanism to avoid the proliferation and activation of T cells with TCRs that bind peripheral self-antigens. This process is known as *peripheral tolerance*, or *T-cell anergy*.¹⁷ It occurs when T cells encounter and bind an antigen that is present in relatively low concentrations and simultaneously fail to receive secondary stimulation from an APC, thereby activating apoptosis.^{18,19} Although T-cell anergy is far from being fully understood, it is apparent that the state of anergy does not always lead to apoptosis, but rather it may lead to a state of reduced ability to proliferate and produce lymphokines. Over time, the anergic state declines and must be reinduced to avoid escape of self-reacting T cells.

Although the thymus gland is the major site of T-cell development, thymus-independent T cells develop elsewhere in the body. These cells are especially abundant in the epithelium of the intestinal tract, where more than 50% express the $\gamma\delta$ TCR.²⁰ It has been proposed that gut epithelial cells provide "thymuslike" signaling to enable thymus-independent T-cell maturation.^{21,22}

To understand the mechanism whereby APCs present antigenic molecules to lymphocytes, it is necessary to know about the TCR and a special group of cell surface glycoproteins that form the major histocompatibility complex. These glycoproteins fall into two categories, class I and class II MHC molecules. Class I molecules are expressed in all nucleated cells. Class II molecules are expressed constitutively on B lymphocytes and APCs, and during an inflammatory reaction on other cells as well. The molecular structures of the TCR and the MHC molecules are described in the following sections.

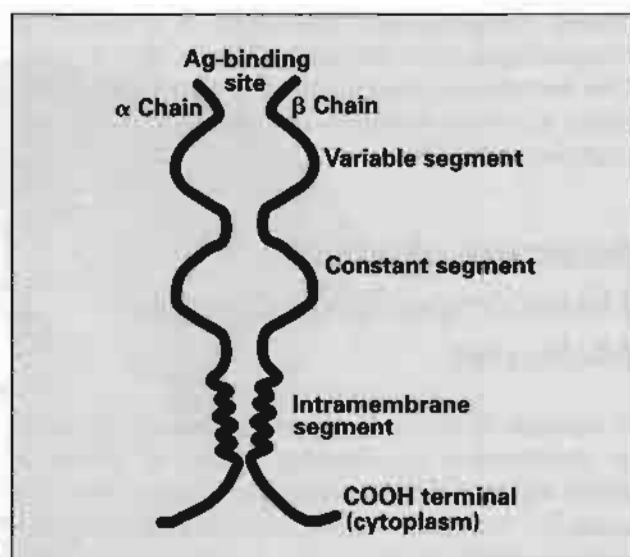


Fig 13-3 Structure of a T-cell antigen (Ag) receptor. The T-cell receptor is a heterodimer consisting of two transmembrane immunoglobulin-like proteins. The amino terminals contain variable domains that cooperate to form the antigen-binding site. Short carboxy terminal domains protrude into the cytoplasm.

Structure of T-Cell Receptors

The T cell recognizes an antigenic peptide by binding it to the TCR. Furthermore, the peptide must be presented on an MHC molecule by an antigen-presenting cell. The TCR comprises two transmembrane polypeptides (Fig 13-3). Each polypeptide contains a constant segment and a variable segment in its extracellular domain. The variable segments of both polypeptide chains cooperate in forming an antigen-binding site, or pocket.²³ Most lymphocytes (85%) have type 2 receptors (TCR2) made up of α and β polypeptides. The remaining lymphocytes have type 1 receptors (TCR1) consisting of γ and δ chains. High numbers of $\gamma\delta$ T cells migrate to the epithelial surfaces of the gut and oral mucosa.²⁴⁻²⁶ Much of the discussion of lymphocytes in this chapter pertains to $\alpha\beta$ TCR-positive lymphocytes.

A subset of human $\gamma\delta$ T cells is stimulated by small, nonpeptide phosphorylated metabolites in a manner that is yet to be fully understood but involves macrophages or other cells capable of generating costimulation. These phosphorylated nonpeptides are generated by bacteria and damaged cells. It has been proposed that nonpeptide-activated $\gamma\delta$ T cells act as sentinels to signal the immune system of the presence of live microorganisms or of cell damage occurring at some site in the body.²⁷ Activated $\gamma\delta$ T cells subsequently perform helper functions by re-

leasing cytokines and interleukins for activation of macrophages, dendritic cells, T cells, and B cells. One well-documented function of $\gamma\delta$ T cells is their ability to induce tolerance to antigens administered orally or inhaled.²⁸

Structure of Major Histocompatibility Complex Molecules

In humans, MHC molecules are encoded by a region of chromosome 6 containing about 200 genes, of which 40 are related to leukocyte function (the HLA system).⁷ The MHC-I and MHC-II (HLA-I and HLA-II) proteins of this system are involved in antigen presentation.

The MHC class Ia molecule consists of a single transmembrane polypeptide with three extracellular domains, $\alpha 1$, $\alpha 2$, and $\alpha 3$ ²⁹ (Fig 13-4). The $\alpha 1$ and the $\alpha 2$ domains are the variable regions, different in every individual. A smaller peripheral membrane protein, $\beta 2$ microglobulin, is associated with the larger transmembrane polypeptide.²⁹ The MHC-Ia molecule is expressed in all nucleated cells. It binds antigenic cytosolic polypeptides that have been transported into the rough endoplasmic reticulum (RER) and presents them to CD8⁺ cytotoxic T cells.

In humans, the MHC class Ia (HLA-I) molecules are encoded by the *HLA* genes (*HLA-A*, *HLA-B*, and *HLA-C*). The human genome also encodes another group of MHC-I-like molecules, the MHC-Ib proteins. These molecules are expressed in relatively high numbers on epithelial cells, where they may engage in presentation of lipid antigens.³⁰

Class II MHC molecules are heterodimers of two transmembrane polypeptides³¹ (see Fig 13-4). The $\alpha 1$ and $\beta 1$ domains are highly variable. They form an antigen-binding groove that recognizes peptides of about 15 to 24 amino acids. Class II MHC molecules are expressed in APCs, thymic epithelial cells, fibroblasts, and B lymphocytes. In the inflammatory reaction, MHC-II molecules are also expressed on activated endothelial cells and keratinocytes. Type II MHC molecules bind antigenic peptides derived from proteins that have been endocytosed from the extracellular space or the plasma membrane and partially degraded by lysosomal enzymes and present them to CD4⁺ helper T cells. The variable segments of the MHC molecules make it possible to construct an enormous number of different recognition sites, thereby ensuring that all possible antigenic

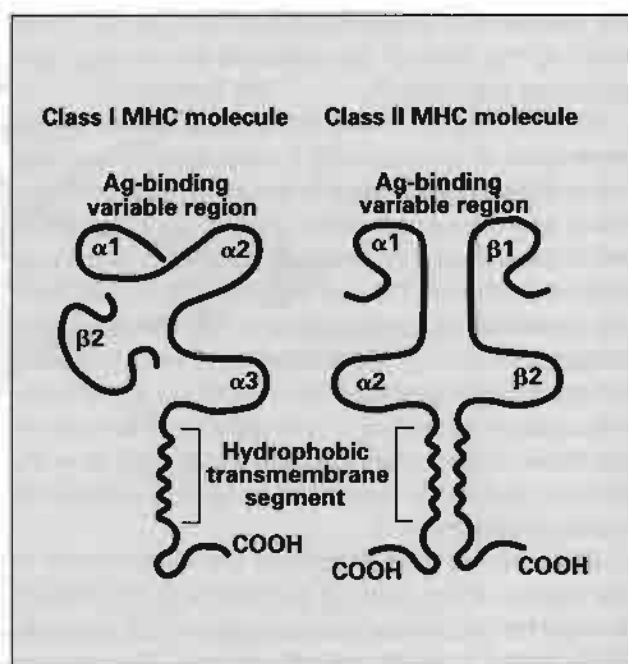


Fig 13-4 Structure of major histocompatibility complex (MHC) molecules. The class I MHC heterodimer consists of one transmembrane protein with two variable segments ($\alpha 1$ and $\alpha 2$) which form the antigen (Ag)-binding site. It associates with $\beta 2$, a peripheral membrane protein. Class II MHC molecules are heterodimers formed by two transmembrane immunoglobulin-like proteins. Each molecule contains variable segments, $\alpha 1$ and $\beta 1$, which together form a single antigen-binding site.

determinants are bound and displayed for potential interaction with a T cell.

Human MHC-II (HLA-II) molecules are encoded by *HLA-DM*, *HLA-DO*, *HLA-DP*, *HLA-DQ*, and *HLA-DR* genes.⁷ Each person expresses about 20 genetically different HLA class II molecules. The *HLA* genes are contained on the short arm of the human chromosome 6. Increased expression of certain alleles of *HLA-DR* genes has been implicated as a risk factor for rheumatoid arthritis and periodontal disease.^{32,33}

Adjacent *HLA* genes on chromosome 6 encode MHC (HLA) class III proteins. The class III molecules include members of the complement system (C2, C4, and factor B), lymphotoxin, and tumor necrosis factor (TNF). The *HLA* genes are also known as *immune response (Ir)* genes.

The MHC molecules form the immunologic basis for distinguishing species and individuals within the species. It is extremely unlikely that two unrelated humans will express similar MHC genetic markers. The genetic rules of MHC inheritance and typing have been reviewed by Goust and Jackson.⁹ The experiments that led to the discovery of the MHC mole-

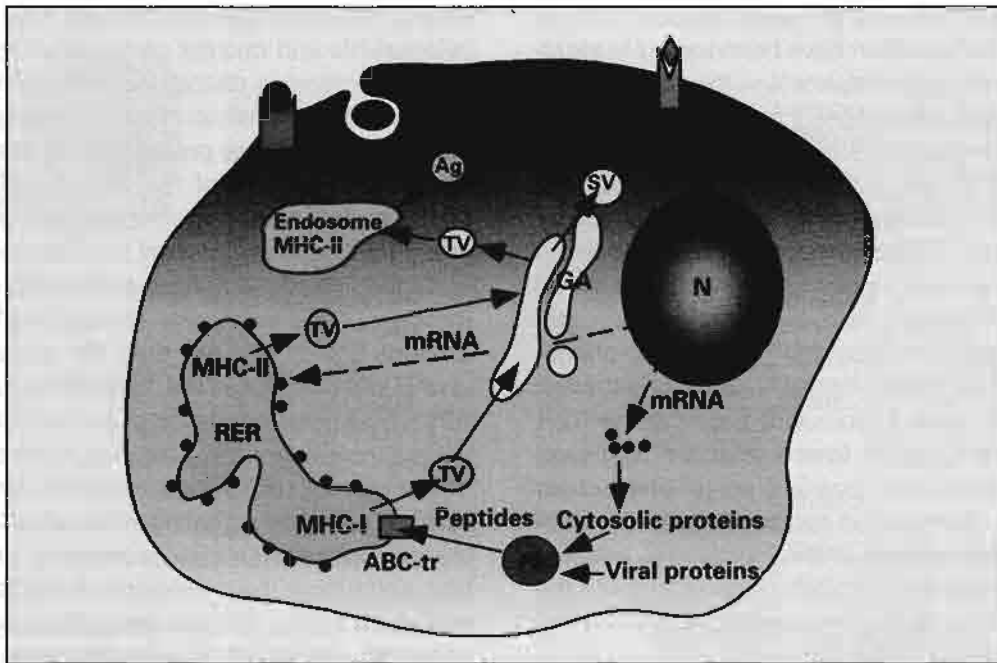


Fig 13-5 Intracellular major histocompatibility complex type I (MHC-I) and type II (MHC-II) pathways for presentation of antigenic peptides. In the MHC-I pathway, cytosolic and nuclear proteins destined for proteolysis are broken down in proteosomes, and the resulting peptide fragments are transported into the lumen of the rough endoplasmic reticulum (RER) by adenosine triphosphate binding cassette transporters (ABC-tr). Viral proteins in infected cells follow the same route. Once inside the RER, the peptides bind to MHC-I molecules that have been manufactured by the RER-ribosomal complex. From the RER, the MHC-I-peptide (MHC-I-P) is transported via the Golgi apparatus (GA) and secretory vesicles (SV) to the plasma membrane. In the MHC-II pathway, the MHC-II molecules are associated with a blocking protein that shields the antigen recognition site from antigenic peptides that have entered the RER via the ABC-tr. The MHC-II molecule leaves the RER to join the endosomal-lysosomal compartment. In that site, the MHC-II molecule loses its shielding protein and becomes available for interaction with antigenic peptides that have been taken in from the extracellular compartment and processed in the endosomal-lysosomal system. (Ag) Antigen; (mRNA) messenger ribonucleic acid; (N) nucleus; (PS) proteosome; (TV) transport vesicle.

cules and their function were described in a Nobel lecture given in 1996 by Zinkernagel.³⁴

Processing and Presentation of Antigenic Peptides

The TCR recognizes (binds) peptide fragments generated through antigen processing. During antigen processing, antigenic proteins undergo limited proteolysis. The resulting peptides are presented on MHC molecules at the cell surface. Two major pathways of antigen processing and presentation have evolved.³⁵ In the first pathway (class I-restricted), cytosolic and nuclear proteins are marked by interaction with ubiquitin and subsequently broken down within large cytoplasmic complexes of proteolytic enzymes, called *proteosomes*^{36,37} (Fig 13-5). Viral pro-

teins synthesized within an infected cell, or any other proteins that gain entry into the cytoplasmic fluid (cytosol), are also destined to undergo destruction in proteosomes.

Peptide fragments released from the proteosomes are transported into the RER by adenosine triphosphate binding cassette transporters located in the membrane of the RER³⁸ (see Fig 13-5). These transporters consist of heterodimers of transporter associated with antigen processing (TAP1 and TAP2) proteins. Class I MHC molecules, synthesized in the RER, acquire the peptide that best fits their antigenic recognition site by interaction with TAP proteins (a process called *antigen loading*).⁷ The MHC-I-peptide complex is then transported from the RER to the Golgi apparatus and subsequently to the plasma membrane in secretory vesicles (see Fig 13-5).

Differences in rates of antigenic peptide loading and cytoplasmic transport have been traced to structural differences (polymorphism) in the non-antigen receptor domains of the MHC-I molecules.³⁹ The foreign peptide fragments displayed at the cell surface on MHC-I molecules act as activating signals for CD8⁺ T cells and as markers for a potential attack by cytolytic T cells. Antigenic peptide presentation on MHC-I molecules is a function of all nucleated cells.

In the second major pathway of antigen processing, proteins are cleaved by proteolysis inside phagosomes and lysosomes. In this class II-restricted pathway, MHC class II molecules made in the RER are temporarily shielded from interaction with peptides by an associated invariant polypeptide chain until they are transported to the endosomal compartment. The invariant chain blocks the antigen-binding site and acts as a sorting signal to route the MHC-II molecules to the endosomal system via the trans-Golgi network⁴⁰ (see Fig 13-5).

Once in the special endosomal compartment, the invariant polypeptide disassociates to uncover the MHC-II-antigen-binding site for interaction with peptides that have been taken into the cell by endocytosis.⁴⁰⁻⁴² Communication with secondary lysosomal compartments also directs phagocytosed peptides to the endosomal compartment. The MHC-II-antigen complexes are subsequently transported to the plasma membrane. Antigen presentation on MHC-II molecules is especially efficient in antigen-presenting (dendritic) cells, B cells, and activated macrophages.⁷ Recent studies have revealed that dendritic cells also have the unique ability to load antigens on naked MHC-II molecules at the cell surface.⁴³

Exceptions to the strict partitioning of the MHC-I and MHC-II pathways have been reported, wherein exogenous antigens escape from phagosomes to gain access to the MHC-I pathway in dendritic cells, macrophages, and B cells.^{44,45}

Cytolytic T cells (CD8⁺) also recognize microbial lipid antigens processed in intracytoplasmic compartments of infected cells. Lipid antigens liberated during microbial killing are presented at the cell surface in association with CD1 molecules. This subset of cytolytic T cells is said to be *CD1 restricted*, a distinct phenotype from MHC-I-restricted cytolytic T cells. During CD1-restricted T-cell activation, the infected target cell is destroyed by a granule-dependent, Fas-independent mechanism.⁴⁶ During this process, the infecting microbes are also destroyed.

Most of the MHC molecules displayed on the cell surface will contain peptides that are not derived from foreign proteins. Most of the class I molecules

display peptide fragments derived from digestion of cytoplasmic and nuclear proteins, while most of the class II molecules display peptides derived from digestion of extracellular matrix molecules, foreign antigens, and soluble proteins of the extracellular fluids. It is estimated that roughly 100,000 to 300,000 MHC-I and/or MHC-II molecules displaying self- and foreign antigens are present on most cells.⁷

Various dendritic antigen-presenting cells located throughout the body are specialized to scavenge proteins and to process them for presentation to T and B cells.^{47,48} Over the long history of evolution, APCs have developed receptors that recognize conserved molecular configurations, such as endotoxin. These pattern recognition receptors facilitate endocytosis, generate signaling molecules that increase the efficiency of antigen processing and presentation, and trigger the expression of costimulatory molecules by APCs. Pattern recognition receptors are especially important in ensuring that an effective adaptive immune response is generated to combat pathogenic organisms.

Prime examples of APCs are the follicular dendritic cells of lymph nodes and the Langerhans cells of skin and oral mucosa. These cells express high levels of MHC-II proteins. Activated monocytes are also potent antigen-presenting cells. Langerhans cells in mucosal epithelium and dendritic cells in mucosal connective tissue act as sentinels in the early detection of foreign antigens. Following the uptake of antigen, these cells migrate into more central locations, such as regional lymph nodes, where they stimulate antigen-specific T-cell proliferation. Because of their efficient antigen-trapping properties and their capacity to generate strong costimulatory signals, APCs play a key role in the primary immune response.⁴⁸

Activation of T Cells

Naïve T cells circulate from the thymus to lymphoid organs, where they undergo activation (antigen-specific priming) during interaction with APCs. Activation of the T cell is a multistep process involving close interaction with APCs (Figs 13-6 and 13-7). The T cell must bind to the APC, recognize antigen presented by the APC, and finally interact with the APC to generate costimulatory signals.⁴⁹

Naïve T cells require approximately 20 hours of MHC-antigen-TCR contact with APCs to achieve the primed state. In contrast, primed cells can become fully active effector cells following a 30-minute APC interaction because of the prior recruitment and as-

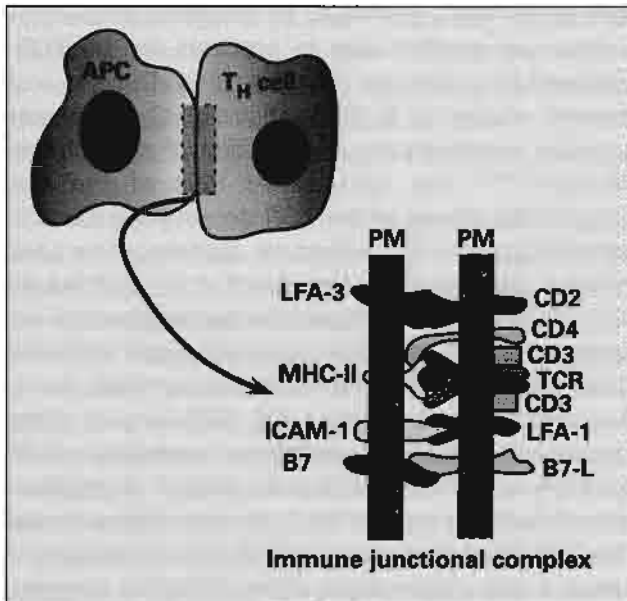
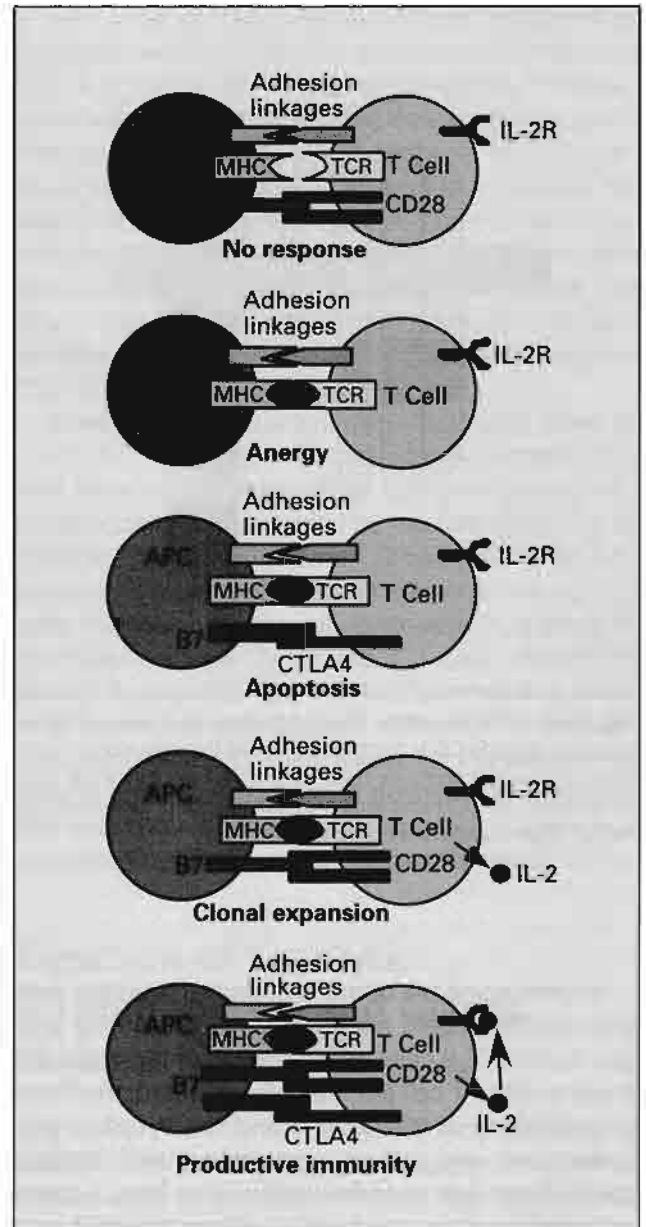


Fig 13-6 Representative cell-to-cell associations (receptor-ligand interactions) during helper T (T_H)-cell activation by an antigen-presenting cell (APC). Specificity of activation is provided by the formation of the T-cell receptor-antigen-major histocompatibility complex (TCR-Ag-MHC) complex, along with signals generated by the activation of the CD4 and CD3 transmembrane proteins. Secondary binding pairs, such as leukocyte function antigen 3-cluster of differentiation 2 (LFA-3-CD2), intercellular adhesion molecule 1-leukocyte function antigen 1 (ICAM-1-LFA-1), and B7-B7 ligand (B7-B7-L), provide essential costimulatory signals that lead to the upregulation of interleukin 2 production and the activation of gene transcription. (PM) Plasma membrane.

Fig 13-7 Summary of some of the most significant receptor-ligand signaling interactions, which can lead either to clonal expansion and productive immunity or to anergy and apoptosis. (Ag) Antigen; (APC) antigen-presenting cell; (CTLA4) cytotoxic T-lymphocyte antigen 4; (IL-2) interleukin 2; (IL-2R) interleukin 2 receptor; (MHC) major histocompatibility complex; (TCR) T-cell receptor. (Adapted from Schultze et al¹⁹ with permission from Elsevier Science.)



sembly of appropriate signaling complexes.⁴⁹ Memory T cells appear to be activated at lower antigen doses and with fewer costimulatory requirements.⁵⁰ Fully activated T_H cells fall into two broad categories, T_H1 and T_H2 lymphocytes, based on their repertoire of secreted cytokines.

Following priming, T cells downregulate those specific chemokine receptors that are used in traversing high endothelial venules (HEVs) and upregulate peripheral and inflammatory chemokine receptors to ensure that they will enter peripheral tissues.⁴⁹ Primed cells proliferate and subsequently home to

peripheral normal and inflamed tissues and to B-cell compartments of lymphoid follicles.^{4,5}

A subset of the primed T cells develops into effector memory T cells that circulate through the body, homing to various tissues. Effector memory T cells are small resting lymphocytes characterized by the expression of the RO isoforms of CD45, high levels of CD44 adhesion protein, and low levels of activation markers such as the IL-2 receptor (CD25).⁵⁰ On reencountering specific antigenic peptide as presented on MHC molecules, memory T cells are activated into effector (cytokine-secreting) cells.

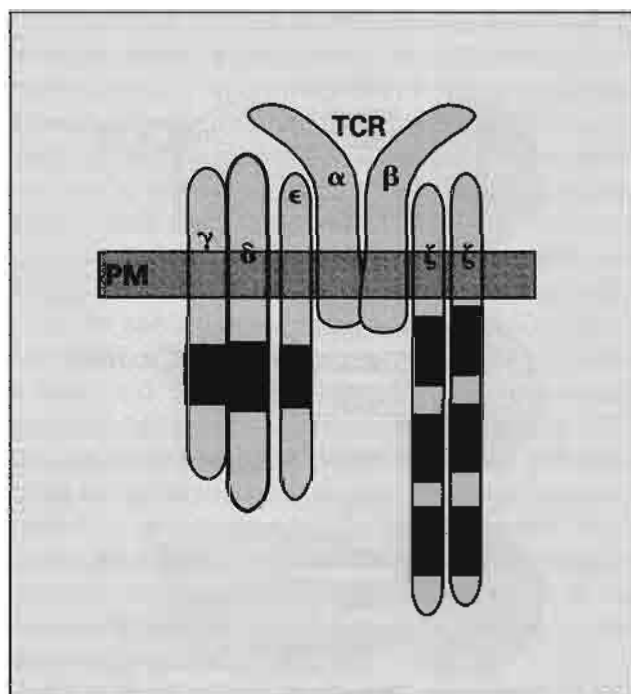


Fig 13-8 Components of the T-cell receptor–cluster of differentiation 3 (TCR-CD3) complex. The five transmembrane proteins of the CD3 complex have longer carboxy terminals than do those of the TCR and thus are available for cytoplasmic signaling. (PM) Plasma membrane.

In developing the humoral immune response, antigen-specific B cells act as APCs in presenting antigen to CD4⁺ T_H2 cells. In the ensuing interaction, the B cell and the T cell are mutually activated, the T cell in production of interleukins and the B cell in production of antibody as a plasma cell.⁵¹ Special chemokines and chemokine receptors have evolved on T and B cells to ensure that antigen-activated cells rendezvous in lymphoid follicles.⁴⁻⁶ The simultaneous interaction of numerous cell surface molecules with their appropriate ligands is essential at every step. These cell surface receptor-ligand interactions trigger the cytoplasmic signaling pathways that lead to T-cell activation. Failure to generate appropriate costimulatory signals leads to anergy, a state of unresponsiveness that may lead to cell death. Cell death may also be triggered by prolonged dendritic cell costimulation.⁴⁹

Integrin molecules have an important role to play in the adhesion of T cells to APCs by forming binding pairs, such as leukocyte function antigen 1 (LFA-1) to intercellular adhesion molecule 1 (ICAM-1) and very late activation 4 (VLA-4) to vascular cell adhesion molecule (VCAM).⁵² Close juxtapositioning of the T

cell to the APC, stabilized by integrins, allows the antigen recognition step to proceed via the TCR-antigen-MHC complex (see Fig 13-6). Dimeric and trimeric clustering of TCR-antigen-MHC complexes appears necessary for the initiation of signal transduction.⁵³⁻⁵⁵ The cytoplasmic tails of the two polypeptide chains of the TCR do not have the ability to trigger the full enzymatic signaling cascades needed for activation. This function is provided by CD3, CD4, and CD2 polypeptide aggregates that are associated with the TCR molecule. Each CD3 unit comprises five transmembrane polypeptides: the γ , δ , ϵ , and two ζ polypeptides (Fig 13-8). Binding of the appropriate antigenic peptide (as presented on an MHC molecule of the APC) in the antigen recognition site of the TCR causes the T cell to polarize toward the APC.^{56,57} Antigen binding also causes changes to occur in the polypeptides of the CD3-CD4 complex that lead to phosphorylation of several cytoplasmic signaling proteins.

Additional receptor-ligand interactions between the T cell and the APC are required for full activation. In T_H cells, the transmembrane molecule CD4 must bind to the MHC-II molecule of the APC (see Fig 13-6). Activation of CD4 by its interaction with MHC-II leads to the phosphorylation of p56^{lck}, a tyrosine protein kinase.⁵⁸ A similar activation of p56^{lck} by CD8 to MHC-I interaction has been reported.⁵⁹ In the activation of cytolytic T cells, the CD8 molecule interacts with the TCR-antigen-MHC-I complex by binding to the $\alpha 3$ domain of MHC-I. The signal transduction events, as they are currently understood, are reviewed briefly under "Basic Science Correlations: Signal Transduction in Lymphocyte Activation."

Costimulatory signaling pairs include CD2 and LFA-1 (CD11a and CD18) in the T cell and their respective receptors, LFA-3 and ICAM-1, on the APC^{10,60} (see Fig 13-6). Potent activating molecules expressed on APCs (including B cells), the B7-1 and B7-2 proteins, have been shown to have key costimulatory roles in T-cell differentiation.^{19,48} Two ligands, CD28 and cytotoxic T-lymphocyte antigen 4 (CTLA4 [CD152]), for the B7 (B7-1 [CD80] and B7-2 [CD86]) molecules have been shown to act as switches for increasing and decreasing the T-cell response^{18,61,62} (see Fig 13-7).

Binding of B7 to T-cell CD28 stimulates T cells to produce cytokines, especially IL-2 and its receptor, and to increase the expression of CD40-ligand, leading to increased cell proliferation and survival. In contrast, when B7 binds to CTLA4, without simultaneous B7-CD28 interaction, a negative signal results in apoptosis and downregulation of the immune re-

sponse¹⁹ (see Fig 13-7). The T cells may require re-stimulation from APCs via the B7-CD28 axis to continue to express cytokines in local sites of inflammation.⁶³ Signaling mediated by B7-1 favors T_H1 cell development, while B7-2 signaling favors the T_H2 type response.

Circulating naïve and memory effector T cells tend to be small cells with a high nucleus-to-cytoplasm ratio. During the activation of T cells, there is an increase in both nuclear and cytoplasmic volume. As more DNA becomes metabolically active, the nucleus assumes a more euchromatic appearance, and the nucleolus becomes more evident. The cytoplasmic content of polyribosomes rises dramatically in T_H cells, while numerous dense granules are developed in cytolytic T cells. It is difficult, if not impossible, to identify T cells from B cells in tissue sections unless special immunologic stains specific for cell surface differentiation markers are used.

Function of Helper T Cells

The major function of T_H cells is to produce lymphokines (cytokines) that help to stimulate plasma cell (B-cell) development and activate other inflammatory cells⁶⁴ (discussed in "Activation of B Cells," later in this chapter). Newly developed (virgin) T_H cells produce low levels of IL-2. This cytokine sustains the survival of T cells. Activated T cells produce more IL-2 and express additional IL-2 receptors and cytokines, including IL-4, IL-5, IL-10, and interferon γ (IFN- γ).⁶⁴

During inflammatory reactions, T_H cells undergo additional maturation into two broad categories (a process called *polarization of T cells*): T_H1 cells, which produce mainly IL-2, TNF- β , and IFN- γ , and T_H2 cells, which produce IL-4, IL-5, IL-6, IL-10, and IL-13.⁶⁵ In conditions where the T_H1 cells predominate, the inflammatory infiltrate is richly populated with CD8⁺ T cells and macrophages (cellular immunity). On the other hand, T_H2 lesions contain many CD4⁺ T cells, mast cells, plasma cells, basophils, and eosinophils, all cells that are stimulated by IL-4, IL-5, and IL-6.

Polarization of T cells is regulated by exposure to cytokines derived from dendritic cells and many other cell types.⁶⁶ Thus, the initial dendritic cell interaction with antigen appears to regulate T_H cell polarization through the expression of specific interleukins.⁶⁶ Myeloid dendritic cells (DC1s) appear to have significant roles in secreting cytokines, such as IL-12, that regulate T_H1 cell survival.^{67,68} On the other hand, lymphoid dendritic cells (DC2s) promote T_H2 cell and plasma cell differentiation.^{68,69}

The types of chemokines that are generated locally also heavily influence selective concentration of T_H1 or T_H2 cells in areas of inflammation.⁷⁰ Effector cells such as monocytes and macrophages share chemokine receptors similar to those of T_H1 cells; thus, they tend to localize to the same tissues. Basophils and eosinophils express chemokine receptors similar to those found on T_H2 cells. Neutrophils respond to a different group of chemokines. It is also known that T_H1 cells express more selectins, thereby providing T_H1 cells a greater chance of extravasation than T_H2 cells.⁷¹

Interleukin 12 and interleukin 4 stimulate development of T_H1 and T_H2 cells, respectively.⁷² Interferon γ also induces T_H1 polarization. The polarization of T_H types is partially maintained by the fact that IFN- γ inhibits the proliferation of the T_H2 cell type, while IL-10 tends to decrease the production of IFN- γ by T_H1 cells. Interleukin 10 also acts to block costimulatory interactions between APCs and T_H1 cells, thereby inducing T_H1 anergy. Interleukin 12 promotes T_H1 survival, prolonging cell-mediated immunity.⁷³ In contrast, IL-4 promotes the T_H2 -type lesion.^{74,75} Periodontal disease is a primarily T_H1 lesion during its early stages, and in its chronic condition becomes a T_H2 -type lesion populated by large numbers of plasma cells.^{25,76,77}

Function of Cytolytic T Lymphocytes and Classic Natural Killer Cells

The primary function of cytolytic T lymphocytes and natural killer (NK) cells is to engage and destroy tumor cells and cells infected by intracellular pathogens such as viruses, bacteria, and parasites. Recent studies have focused on the ability of antigen-activated cytolytic T lymphocytes to regulate immune responses to pathogens by secreting a wide spectrum of chemokines.⁷⁸ The CD8⁺ lymphocytes are now recognized as a major source of chemokines that regulate leukocyte traffic to pathogen-infected sites. By virtue of their capacity to interact with antigen-MHC-I complexes, the cytolytic T lymphocytes can be activated by a wide spectrum of cells that display the cognate foreign antigen.⁷⁸ This makes them ideal sentinels for initiating a local inflammatory response to pathogen-infected cells by establishing a chemokine gradient for other classes of lymphocytes, neutrophils, and macrophages.

The naïve cytolytic T lymphocyte is a small lymphocyte (CD8⁺) without a complement of granules.

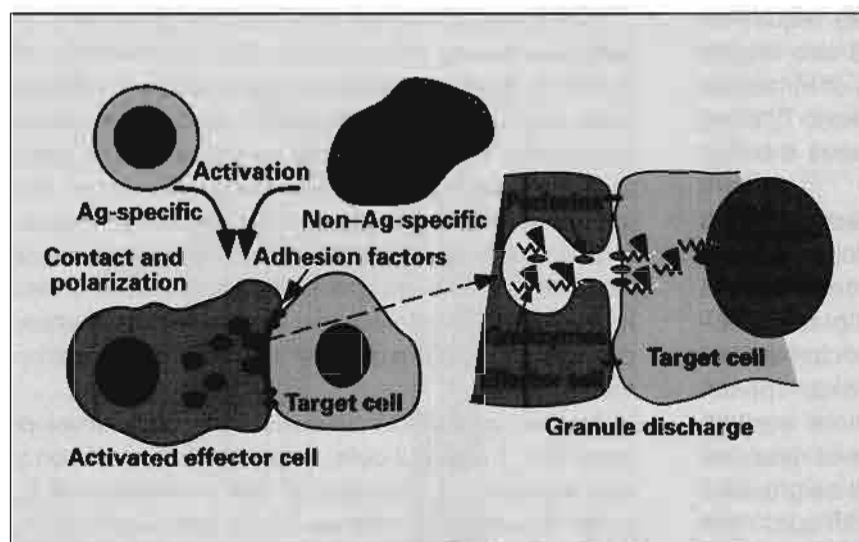


Fig 13-9 Target cell destruction by cytotoxic T cells (CTC) and natural killer (NK) cells. Cytotoxic T cells must be activated by encounters with specific antigens (Ag) expressed on the target cells. Natural killer cells select target cells by non-antigen-specific recognition events. Both cell types develop polarity, transporting granules (Gr) toward the target cell. Exocytosis of perforins and granzymes into the narrow intercellular space between the effector cell and the target occurs following contact. Granzymes gain access to the target cell cytoplasm by diffusion through aqueous channels formed in the plasma membrane by perforin molecular complexes. The granzymes activate interleukin 1 β -converting enzymes, inducing DNA fragmentation and apoptosis.

On activation by contact with antigen (TCR-antigen-class I MHC bridge), and appropriate cytokine stimulation, the cytolytic T lymphocyte undergoes hypertrophy and produces special cytoplasmic protease-containing granules and chemokines. Thus, target cell killing by cytotoxic T lymphocytes is antigen specific.

Natural killer cells are members of the large granular lymphocyte pool that form part of the body's innate defense system. Because they lack T-cell antigen receptors and the CD3 complex, they are activated through other signaling pathways, including initiation by IFN- α and IFN- β , and by engagement of their Fc receptors by antibody.^{79,80} Natural killer cells can be activated through engagement of their Fc receptors, thereby attacking and destroying cells that have become covered by antibody, in a process known as *antibody-dependent cellular cytotoxicity*. Many other cells of the body, such as macrophages, monocytes, and neutrophils, are also capable of antibody-dependent cellular cytotoxicity; however, the NK cell is highly specialized for this function. Natural killer cells may also become activated following binding of bacterial lipids on CD1 molecules.⁷⁹

Natural killer cells can attack and destroy tumor cells and virus-infected cells that have become coated with antibody and/or ligands for a newly discovered class of NK-activating receptors, the natural cytotoxicity receptors such as NKp46.⁸¹⁻⁸³ Low NK cell activity is associated with increased susceptibility to viral infections and certain types of cancer. Recent studies have also discovered NK cell receptors (such as NKp46) that recognize viral hemagglutinins dis-

played on virus-infected cells. Destruction of virus-infected cells appears to occur by combined cytolytic T lymphocyte- and NK-mediated cytotoxicity and the effects of increased expression of IFN- γ .^{78,79}

Normal cells must be protected from being killed during chance encounters with NK cells. Recent reports have described inhibitory receptors in NK cells that engage MHC-I molecules on presumptive target cells.⁸⁴ The activation of the NK cell is inhibited when the NK inhibitory receptors in the plasma membrane of the NK cell engage self-MHC-I molecules in the surface of a normal healthy cell. The cytoplasmic terminal of the inhibitory receptor contains a tyrosine-based inhibition motif that undergoes phosphorylation during binding of MHC-I, allowing it to activate Src-like tyrosine phosphatase. This blocks NK cell action by countering the actions of tyrosine kinases.

In contrast, NK cell killing occurs following activation of natural cytotoxicity receptors by glycoproteins (for example, the hemagglutinins on virus-infected cells) on the target cell, while simultaneously failing to receive an inhibitory signal from MHC-I engagement. Tumor cells and virus-infected cells typically express reduced levels of MHC-I molecules.⁸²

Two mechanisms of cytotoxicity operate side-by-side in effecting the death of the target cell. These are the granular-perforin pathway and the Fas-Fas ligand pathway.^{85,86} Both CD8⁺ cytolytic T cells and NK cells use the perforin-granzyme system to attack and kill their targets.⁸⁶ Following contact with its target, the effector cell establishes cytoplasmic polarity (Fig 13-9). The Golgi apparatus and secretory granules become oriented toward the target cell. Close

contact between the effector cell and its target activates tyrosine kinases and a cascade of cytoplasmic events, leading to the discharge of granule content into a narrow intercellular gap. The cytolytic T lymphocytes and NK cytoplasmic granules contain lytic enzymes (granzymes), toxins, and special molecules (perforins) that can form channels through the plasma membrane of the target cell.^{87,88} Stable contact of the interacting cells is assisted by integrin (LFA-1)-binding interactions.¹⁰

The insertion of perforins in the target cell plasma membrane creates pores through which granzymes and toxins pass into the interior of the target cell. The granzymes are serine esterases that cleave and activate proteases (caspases) belonging to a family of interleukin 1 β -converting enzymes.^{88,89} These enzymes have the capacity to induce DNA fragmentation and apoptosis of the target cell.

The second effector pathway of cytotoxicity involves the Fas antigen (CD95), a cell surface transmembrane protein with homology in its extracellular domain to the epidermal growth factor and TNF receptors.⁸⁸ The presence of Fas antigen in the plasma membrane renders a target cell susceptible to effector cells that express the Fas ligand. The Fas ligand is a transmembrane protein that engages three Fas molecules to trigger a signaling complex in the target cell that activates the apoptotic suicide pathway via interleukin 1 β -converting enzyme protease (caspases) activity.

Both Fas and Fas ligand are upregulated in activated T cells, thereby creating a condition for the apoptotic downregulation of the T-cell response. When cells receive a TCR-CD3 signal concurrent with Fas-Fas ligand signals, they enter the apoptosis pathway. Additional mechanisms for downregulating the immune response by apoptosis of activated T cells include triggering by CTLA4 (see Fig 13-7), TNF receptor, and anti-HLA class I (MHC-I) antibodies.¹⁹

Development of B Lymphocytes

Immature (virgin) B cells originate from stem cells (B-cell progenitors) in close relation to stromal cells of the bone marrow. Soluble signaling molecules of stromal cell origin regulate primary B-cell development, involving cell proliferation, differentiation, and apoptosis, in the bone marrow compartment. After a period of development involving several rounds of cell division, mature B cells expressing surface immunoglobulin M (IgM) and immunoglobulin D (IgD) enter the blood circulation. Any B cells expressing

high-affinity antibody to self-antigens are aborted in the bone marrow.

The life span of a newly differentiated B cell is only a few days, unless it is triggered by antigen to undergo memory cell or plasma cell precursor differentiation. It has been estimated that the rate of B-cell production in the bone marrow is high enough to replace the entire peripheral B-cell population in several days.⁹⁰

Two major classes of B cells have been identified. The first to develop are B1 cells (fetal). They express CD5, a surface adhesion molecule, and surface IgM antibodies. Class B1 cells populate the peritoneal and pleural cavities and the spleen, where they differentiate into plasma cells without the help of T cells to produce natural antibodies.^{2,91} These IgM and immunoglobulin A (IgA) antibodies react with several antigens typically present on bacterial pathogens. They form part of the innate immune system, important in protecting mucosal tissues. Some IgM antibodies are also reactive with self-antigens.

The second class of B cells, the B2 cells, develop in adult bone marrow. They lack CD5 and require the help of T cells to differentiate into plasma cells that secrete immunoglobulin G (IgG), or IgA, IgD, and immunoglobulin E (IgE) antibodies directed at specific antigens.^{2,91} Type B2 cells home to secondary lymphoid tissues, such as lymph nodes and spleen, where they interact with dendritic cells to form germinal centers.

The B2 cells are activated in perifollicular locations of secondary lymphoid tissues through interaction with antigen-specific CD4⁺ T cells or interdigitating dendritic cells. In lymph nodes, antigen-activated T and B cells migrate toward each other in response to various chemokines. Both cell types meet and interact at the margins of the lymphoid follicles.⁴⁻⁶ Following successful interaction, requiring costimulation through interleukins and C40-C40 ligand interaction, the B2 cells enter the follicular zone as centroblasts (germinal center founder cells).^{92,93}

Within the germinal center, a single B-cell blast will divide rapidly over 3 to 4 days to produce numerous centroblasts. Hypermutations in the genes encoding the variable regions of antibody molecules occur during these cell divisions. The centroblasts mature into centrocytes, reexpressing surface immunoglobulin. Shortly thereafter, centrocytes undergo a selection process wherein only those centroblasts that express antibody of the highest specificity and affinity for the stimulating antigen are permitted to survive.

In the classic view of germinal center structure, centrocytes move toward the middle of the germinal

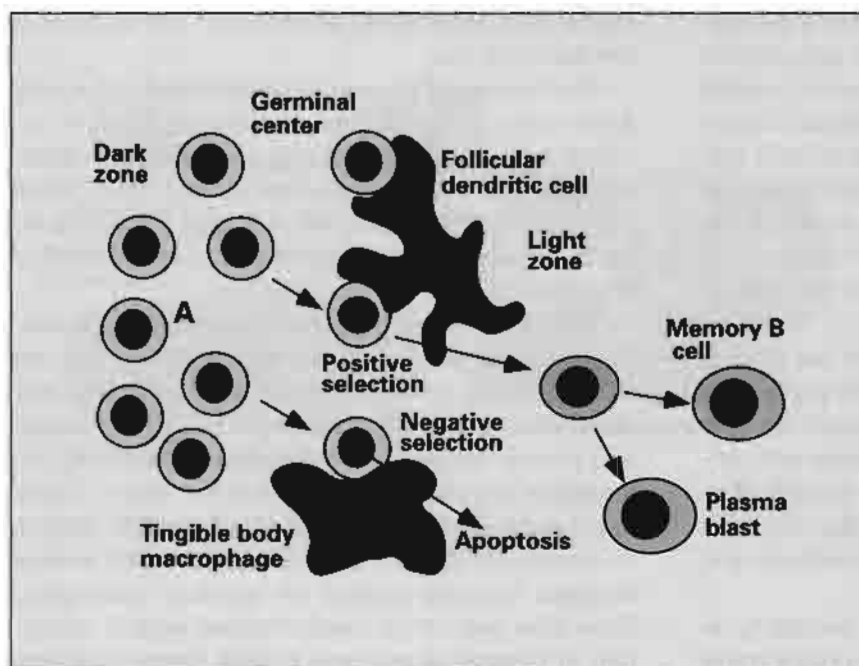


Fig 13-10 Steps in the differentiation of B cells in lymph node germinal centers. Activated B cells (A) proliferate along the peripheral region of the follicle and migrate inward (centrocyte) for interaction with tingible body macrophages and follicular dendritic cells. Positive interaction between high-affinity antibody-producing B cells and antigen-presenting cells leads to further maturation into memory cells and plasma cells. The B cells that are not selected for survival undergo cell death and are destroyed by macrophages.

center, where they interact with follicular dendritic cells⁹⁴ (Fig 13-10). Recent analysis of germinal centers suggest that they represent dynamic structures with different spatial distributions of follicular dendritic cells, centroblasts, and centrocytes, depending on the type of lymphoid tissue and the past history of exposure to a specific antigen.⁹⁵ Trafficking and localization of T and B cells within secondary lymphoid tissues and lymphoid follicles is controlled by specific chemokines and chemokine receptors expressed on T and B cells and on endothelial and stromal cells.⁴⁻⁶

The follicular dendritic cell has the capacity to bind and retain intact antigen on its plasma membrane over long periods of time.⁹⁶ Antigen-antibody complexes are trapped by follicular dendritic cells during secondary immune responses.⁹⁷ Follicular dendritic cells also express ICAM-1 and VCAM-1, which form adhesion sites for integrins expressed on the B cells.

During successful binding to a B cell, the follicular dendritic cell expresses increased amounts of CD40 ligand, a potent stimulator of B-cell survival.⁹⁸ In addition, T cells that express CD40 ligand are present in the germinal center, forming part of the B-cell-follicular dendritic cell clusters. The costimulatory signals generated via the CD40-CD40 ligand, LFA-1-ICAM-1, and VLA-4-VCAM-1 binding events ensure that the centrocyte will survive to become a memory

B cell. In the absence of CD40 costimulation, the centrocytes become secondary B-cell blasts (plasma cell precursors).⁹³ Interleukins 12 and 4, key factors in B-cell survival and differentiation, are produced by dendritic cells and activated T cells following CD40 engagement.⁹⁹

Memory cells may remain in germinal centers; however, most circulate to peripheral tissues. They have a large euchromatic nucleus and a small amount of cytoplasm containing many free ribosomes. Based on DNA labeling studies, it is estimated that peripheral memory B cells are long-lived cells whose survival is governed by random events occurring in their immediate microenvironment, such as cell-to-cell interactions and levels of cytokines.¹⁰⁰

Centrocytes that fail to bind with high affinity to the dendritic cells undergo apoptosis in the germinal center (see Fig 13-10). The cell death pathway is triggered by a Fas (B-cell)-to-Fas ligand (T-cell) interaction. Fragments of the dead cells are phagocytosed and digested by macrophages. These macrophages contain many large, dense bodies (tingible bodies) that contain residual organic material.

Plasma cell precursors and memory B cells must interact with T_H2 cells to develop into mature plasma cells.⁵¹ Antigen-antibody complexes formed at the cell surface of the B-cell-plasma cell precursor, or taken up from dendritic cells, undergo endocytosis and are processed in special MHC class II compart-

ments of the endosome-lysosome system.^{10,97} The resulting antigenic peptides are displayed on MHC-II molecules of the plasma cell precursor (B cell), where they are available for binding to the appropriate TCR of a T_H2 cell. This T_H2 -cell-B-cell cooperation results in the production of IL-2, IL-4, and IL-6 by the T_H cell.⁶⁴ These cytokines stimulate the maturation of the plasma cell. In this case, the plasma cell precursor (B cell) acts as the antigen-presenting cell in its interaction with the helper T cell.

Activation of B Cells

The B-cell antigen receptor (BCR) consists of membrane-bound antibody and associated signaling proteins (Fig 13-11). The B cells do not engage antigenic peptides presented on MHC molecules. Instead, they bind complete antigen proteins on the antibody component of the BCR. The heavy chains of the BCR antibody molecules do not extend into the cytoplasm and thus are unable to generate activation signals. Two additional transmembrane proteins with long cytoplasmic tails, Ig- α (CD79a) and Ig- β (CD79b), are associated with the transmembrane antibody molecule.¹⁰¹ Each BCR associates with two Ig- α and Ig- β heterodimers to form the BCR complex (see Fig 13-11).

The BCR binds unprocessed antigen, generating a relatively weak activating signal, probably via the cytoplasmic tail of the Ig- α protein because it contains amino acid sequences similar to those that are present in the CD3 molecule.¹⁰¹ The BCR and coreceptor signals activate increased expression of various surface proteins, including MHC-II, B7, and cytokine receptors. To produce full B-cell activation and plasma cell formation, various coreceptors in the B-cell plasma membrane, including cytokine receptors, must engage their counterligands, in a process involving helper T-cell cooperation (Fig 13-12). This occurs as the B cell, acting like an APC, interacts with antigen-specific TCRs on T_H2 cells.¹⁰

Antigen processing and presentation is recognized as an important function of B cells. Because they bind antigens on specific surface immunoglobulins (BCRs), they are 1,000 times more efficient than other APCs that must take in antigens in non-specific, bulk-type processes. Following binding of antigen to the BCR, the complex translocates to ganglioside-rich sections of the plasma membrane and is then internalized and directed to the endosome-phagosome compartment for processing and peptide loading of MHC-II molecules.¹⁰² By the very nature of MHC-II antigen processing during B-cell-T-

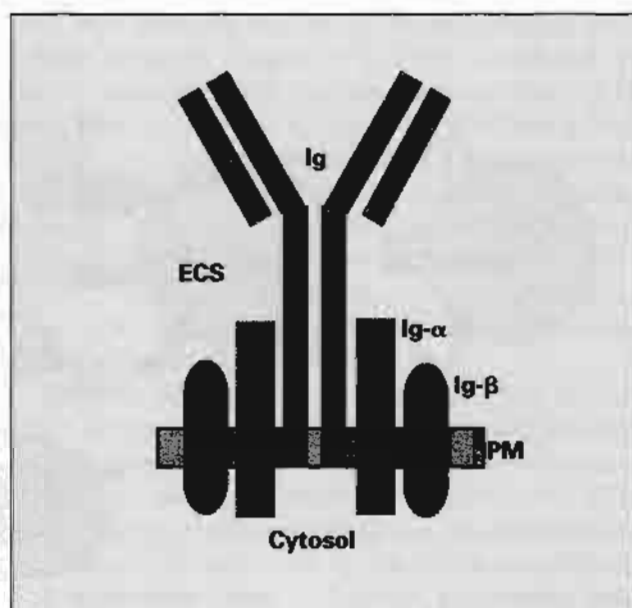


Fig 13-11 Components of the B-cell receptor. The immunoglobulin (Ig) dimer does not protrude into the cytoplasm. Two pairs of transmembrane proteins, Ig- α and Ig- β , associate with the antibody. They have large carboxy terminals in the cytoplasm that carry out signaling interactions with other cytoplasmic proteins following binding of antigen by the Ig receptor. (ECS) Extracellular space; (PM) plasma membrane.

cell interactions, the resulting antibody response will be one of multiple isotypes. Processed antigenic peptide is displayed on B-cell MHC-II molecules for interaction with TCR-CD3-CD4 complexes of the T cell.

Close apposition of the cells occurs during this interaction process. During this interaction, various adhesion linkages that take place have costimulatory functions that further activate the B cell. Cytokine production by the associated T_H cell leads to full development of the plasma cell following disassociation of the two interacting cell types (see Fig 13-12). One of the most potent B2-cell costimulatory signals involves the binding of CD40 ligand (CD154), generated either in an activated T_H cell or an APC. A newly identified member of the tumor necrosis factor family, B-lymphocyte stimulator, plays a role similar to that of CD40 ligand during monocyte-specific activation of B1 cells.¹⁰³

Serum contains a class of proteins, the complement factors, that support and potentiate the immune response in a variety of ways. These factors are more fully described in chapter 14. The complement receptor 2 (CR2) protein (also known as CD21), and the transmembrane protein, CD19, form

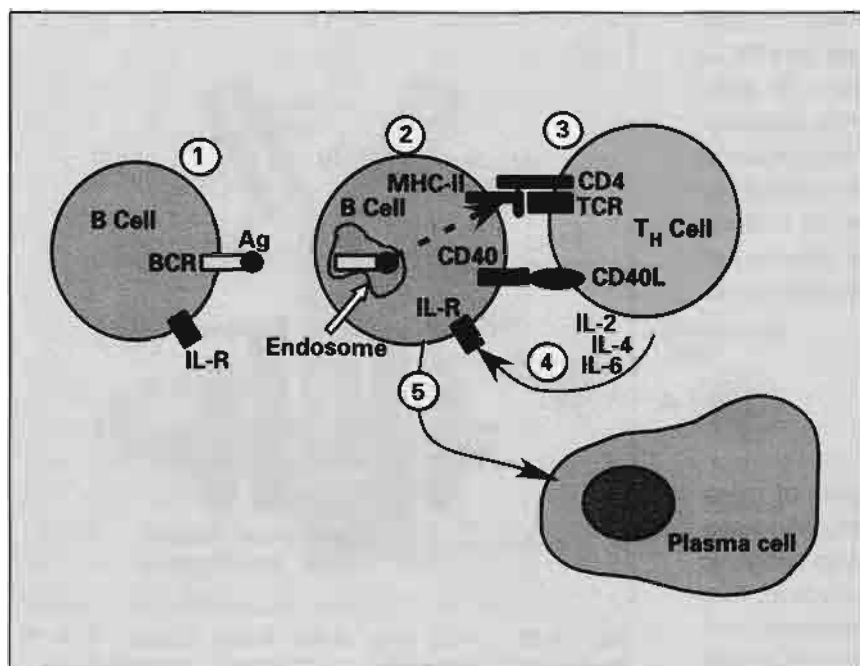


Fig 13-12 Steps in B-cell activation and plasma cell differentiation. (1) Antigen (Ag) binds to the B-cell receptor (BCR), starting the activation process. (2) Antigen is also taken into the B cell and processed for presentation on major histocompatibility complex type II (MHC-II) to an interacting helper T (T_H) cell. (3) Via the association of the antigen-laden MHC-II with the T-cell receptor (TCR)-CD4 complex, and the CD40-CD40 ligand (CD40L) bridge, further activation signals are generated, leading to (4) the production of interleukins (IL). (5) The B cell and its differentiation are released into a plasma cell. (IL-R) Interleukin receptor.

a coreceptor complex that participates in antigen activation of B cells, especially B1 cells that recognize pathogen-associated molecular patterns such as bacterial lipopolysaccharides.^{91,104} The CR2-CD19 coreceptor enhances the immune response to antigens that interact with the complement system.¹⁰⁵

A ligand for CR2, C3d protein, is generated during activation of the alternate complement pathway during inflammatory reactions. The potent activating effect of the CR2-CD19 coreceptor has been demonstrated with fusion proteins consisting of an antigen fused to one or more ligands for CR2. A 1,000-fold greater antibody response resulted when an immunizing antigen was fused to three C3d polypeptides of the complement system.

Antigen-antibody complexes bind complement protein (C3 fragments). These antigen-antibody complexes are trapped by dendritic cells and memory B cells and transported to draining lymph nodes. The presentation of C3 fragments associated with the antigen-antibody complexes provides a more powerful activation signal via the costimulatory C3 complement receptor (CR2) on the naïve B-cell membrane.^{105,106} In this way, complement potentiates the immune response to foreign protein, acting somewhat as a natural adjuvant.

Non-Antigen-Specific Activation of T and B Cells

Both T and B cells can be activated by a non-antigen-specific mechanism involving the binding of pathogen-associated molecular patterns of plant and bacterial origin. Many of these substances are also known as *mitogens* because they stimulate mitosis of lymphocytes. Some mitogens act only on T cells (phytohemagglutinin and concanavalin A), while others such as pokeweed mitogen stimulate both T and B cells. Bacterial lipopolysaccharide (endotoxin) produced by Gram-negative organisms is a potent mitogen for B cells.

Recent studies have led to the discovery of receptors for lipopolysaccharide, the best-studied member of the pathogen-associated molecular pattern class of bacterial toxins. The receptor is a member of the Toll-like family of transmembrane proteins.¹⁰⁷⁻¹⁰⁹ Activation of the Toll-like receptor by lipopolysaccharide stimulates increased secretion of proinflammatory cytokines, such as IL-1 and TNF- α . It also increases the expression of CD80 (B7) a costimulatory molecule essential for lymphocyte activation and proliferation. The resulting inflammatory reaction is designed to defend against the invading organism as part of the innate immune system.

Because pathogen-associated molecular patterns are non-antigen-specific (do not involve the TCR-MHC axis), they elicit a polyclonal response by activating many cell lines with different antigen receptors. Polyclonal activation of T and B cells in response to oral bacterial endotoxin has been reported.^{25,110}

The superantigens, another class of immunogens, are able to bind simultaneously to class II MHC molecules of APCs and to the TCR molecule without engaging the specific antigen-binding site of the TCR.¹¹¹⁻¹¹⁴ Most superantigens are of bacterial, viral, and plant origin. Superantigens stimulate polyclonal T-cell responses by interacting with the β chain of the TCR complex. Polyclonal B-cell activation also occurs during exposure to superantigens through non-specific binding to the MHC-II molecule (on the B cell) and the V β chain of the TCR.

Diseases associated with superantigens include food poisoning, toxic shock syndrome, Kawasaki syndrome, and atopic dermatitis.¹¹³ Common characteristics of these diseases are rapid activation of large numbers of lymphocytes and the release of cytokines in large amounts. Conflicting reports of polyclonal activation of lymphocytes by periodontopathic bacterial superantigens have appeared in the literature.^{115,116}

Development of Immunologic Tolerance

Initial immunologic tolerance (central tolerance) occurs during the purging of all T and B cells that express high-affinity TCRs and BCRs to self-antigens. These cells are eliminated in the thymus and bone marrow, respectively, by clonal deletion involving apoptosis. Peripheral tolerance to antigens is accomplished by^{17,19,117}:

1. Rendering cells anergic by a lack of positive costimulatory signals.
2. Apoptotic cell death induced by negative signals.
3. **The action of suppressor T cells.**

Tolerance to intravenously administered antigens is induced when T and B cells bind antigen but fail to receive an appropriate level of costimulation from APCs or from T_H2 cells in the case of B cells. Activation of the B7-CTLA4 signaling path without concurrent costimulation via CD28 has been shown to induce cell death of T and B cells^{18,19} (see Fig 13-7). Serum contains polyreactive antibodies (IgD) that

bind with relatively low affinity to self-antigens and foreign antigens. They form a small percentage of serum antibodies. Polyreactive antibody-binding B cells have the potential to render T cells tolerant during antigen presentation because the polyreactive antibody-binding B cells lack B7 costimulatory molecules.¹¹⁸ Lymphocyte programmed cell death may also occur by activation of the Fas-Fas ligand pathway.¹¹⁹ In contrast, stimulation via CD2 appears to have a role in counteracting the activation of the cell death pathway in T cells.¹²⁰

Because most antigen encounters occur at mucosal surfaces, an effective tolerance mechanism must be present in those sites to avoid overstimulation of the defense system to nonpathogens. The great majority of antigen exposure in the gut leads to hyporesponsiveness (oral tolerance), a function of T_H3 suppressor cells.¹²¹ Antigen processing and presentation between APCs and T_H3 cells (CD4⁺) occurs in Peyer's patches, while CD8⁺ suppressor cells are probably activated in the epithelial compartment. On reencounter with the activating antigen in peripheral mucosal tissues, the suppressor cells release large amounts of TGF- β and IL-4, which are potent immunosuppressive cytokines.^{117,121} Tolerance following nasal administration of antigen appears to involve activation of immunosuppressor cells in a manner similar to orally induced tolerance.¹²¹

In vivo, the anergic state declines over time and must be continually reinforced. Tolerance to self-antigens is not always 100% effective, and low levels of autoantibodies to certain proteins are measured in serum. These are usually low-affinity antibodies. Autoimmune diseases develop when T and B cells escape anergy and clonal deletion to produce high-affinity IgG directed against self-antigens.

Structure and Function of Plasma Cells

Plasma cells are terminally differentiated mature antibody-secreting cells. Many plasma cells are long-lived, capable of secreting antibody for a year or more.¹²² The secreted antibodies are of the same specificity as the antigen-binding surface immunoglobulin of the plasma cell precursor. Antibodies are transported to the extracellular space in secretory vesicles or are released during the disintegration of dying plasma cells.

Plasma cells have a highly characteristic appearance that makes them easily identifiable in tissue sections. The plasma cell nucleus displays a typical

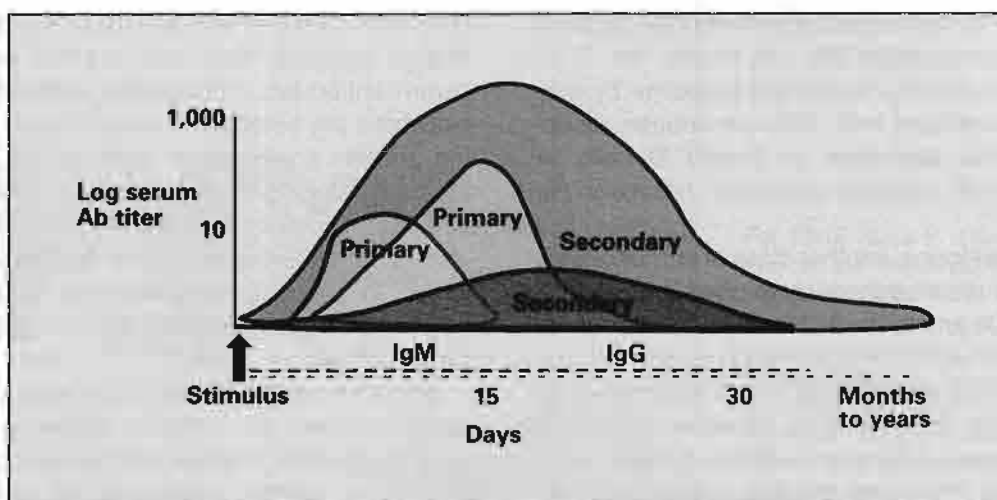


Fig 13-13 Comparison of the primary and secondary antibody (Ab) response. In the primary response, immunoglobulin M (IgM, *pale blue*) rises first after a short delay following antigen stimulus. This is followed by a longer-lasting rise of immunoglobulin G (IgG, *pale green*) to a higher level. In the secondary response to antigen challenge, both IgG (*dark green*) and IgM (*dark blue*) appear more rapidly following the stimulus, with IgG reaching very high levels and for a much longer time than IgM.

“clock-face” pattern of heterochromatin distribution. The cytoplasm contains a well-developed Golgi complex and a large complement of RER. Because of the extensive system of RER, the cell is stained intensely with basophilic dyes. The Golgi complex appears as a pale, lightly stained area adjacent to the nucleus.

Plasma cells are plentiful in bone marrow, the medullary cords of the spleen, the medullary region of lymph nodes, and in the lamina propria of mucosal tissues. They form a significant component of the infiltrate in chronic inflammatory lesions, such as in long-standing gingivitis and periodontitis. Long-lived plasma cells contribute to “immunologic memory” by maintaining long-lasting specific antibody titers in serum.^{122,123}

Plasma cells produce five classes of immunoglobulins: IgG (75%), IgM (10%), IgA (15%), IgD (less than 1%), and IgE (less than 1%). Serum immunoglobulin is mainly derived from the secretions of plasma cells in the lymph nodes and spleen. About 5% to 15% of the total circulating pool of lymphocytes is composed of B cells. Naïve circulating B lymphocytes bear IgM or IgD antigen receptors. The IgG-, IgE-, and IgA-bearing B cells are found in specific tissue locations; for example, the IgA-positive cells concentrate beneath mucosal surfaces and in the minor salivary glands.

Initiation of Serum Antibody Response

In response to a systemic antigen challenge, a wave of serum antibody is produced by plasma cells within the major lymphoid organs. After a lag phase of 3 to 4 days, antibody begins to appear in the circulation.¹²⁴ The titer of antibody rises exponentially for several days, peaks at about 10 days, and subsequently declines (Fig 13-13). Immunoglobulin M antibodies appear first, but do not reach the same level as the IgG antibodies. Antigenic challenge to mucosal lymphoid tissue produces a predominantly IgA response.

A fundamental aspect of the acquired immune response is the enhanced production of serum antibody to a second antigen challenge. During the initial or primary antigen challenge, numerous memory B cells (as well as memory T_H cells) are developed. Memory cells prime the system to produce a larger pool of plasma cells in response to a secondary exposure to the antigen. In the secondary response, after a shorter lag phase, the level of IgG antibodies rises rapidly and steeply to about 100 times higher than in the primary response and thereafter declines more slowly (see Fig 13-13). The level of IgM in the secondary response is less than that in the initial response. A secondary response is also called an *anamnesic response*.

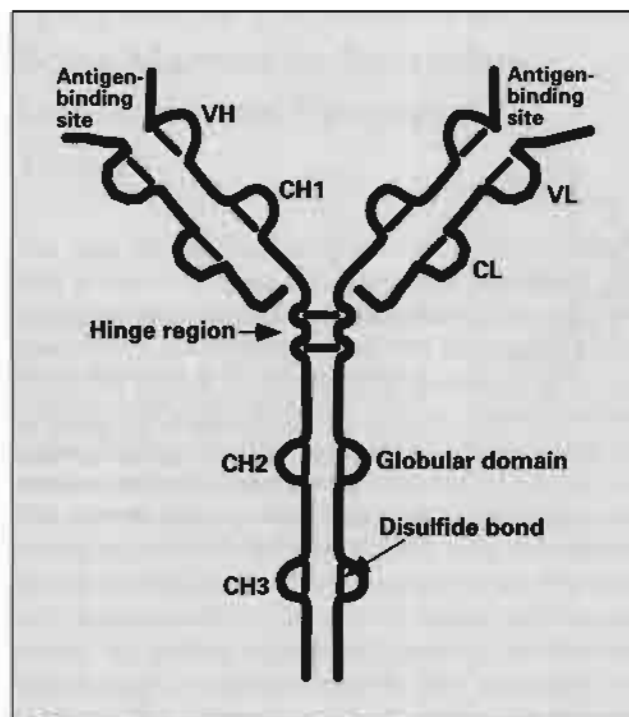


Fig 13-14 Structure of the immunoglobulin G antibody. The complex consists of four polypeptide chains (two heavy [H] and two light [L] chains) held together by disulfide bonds. The heavy chains have three constant (CH1, CH2, and CH3) and one variable (VH) globular domains. The two light chains have a constant globular domain (CL) and a variable domain (VL). Antigen-binding sites are formed by the juncture of the VL and VH domains.

During antigen challenge, the initially high level of antibody is the result of secretion by short-lived plasma cells. This is followed by sustained antibody secretion over longer periods of time by a subpopulation of long-lived plasma cells.¹²²

Antibody structure

Antibodies (immunoglobulins) are specialized glycoproteins present in the serum, tissue fluids, and mucosal secretions and on the surface of B lymphocytes. There are five subclasses of immunoglobulins: IgG, IgA, IgM, IgD, and IgE. The smallest and least complex is IgG (Fig 13-14). It is constructed of four polypeptide chains: two identical light chains and two identical heavy chains. The light chains and heavy chains are linked by covalent and noncovalent bonds to form the IgG antibody molecule. The heavy chains are expressed in four isotypes, giving rise to the subclasses IgG1 to IgG4. Both light chains and heavy chains contain constant domains and variable domains.

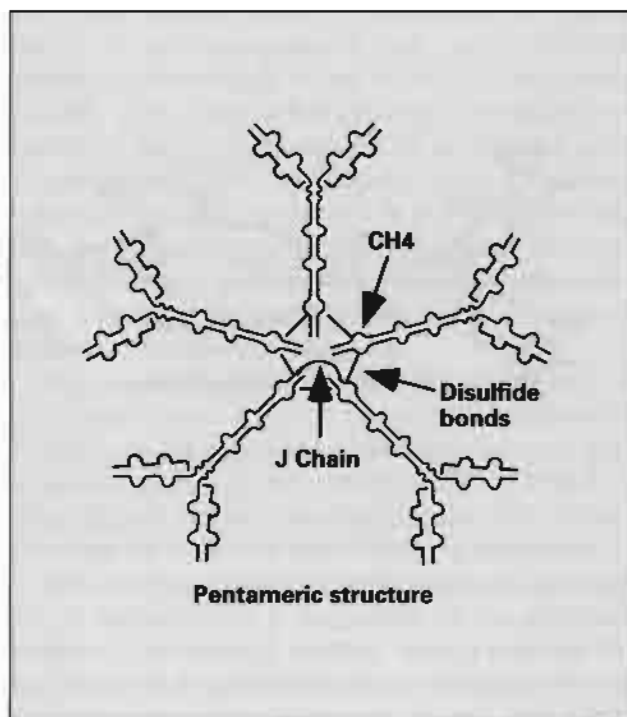


Fig 13-15 Structure of the immunoglobulin M molecule. The complex consists of five immunoglobulin G-like molecules joined by disulfide bonds and a J chain polypeptide. Note the additional constant globular domain (CH4) on the carboxy terminal of the heavy chains.

The variable domains of IgG are located at the amino terminal, where they form two antigen-binding sites (the paratopes). The constant parts of the polypeptides of both light chains and heavy chains contain globular regions that are stabilized by intra-chain disulfide bonds (see Fig 13-14). A flexible hinge region is present on the heavy chain just proximal to the point of linkage to the light chain. The hinge permits movement of the antigen-binding outer segment in relation to the portion of the antibody that is anchored in the plasma membrane. This hinge region is susceptible to enzymatic cleavage, giving rise to antigen-binding fragments (Fab units) and a single non-antigen-binding fragment (the Fc unit). Carbohydrate moieties are also attached to the polypeptides at their constant domains.

The amino acid sequence of the constant domain of the light chain is the same for all five classes of antibody. However all five classes of heavy chain constant domains have different amino acid sequences.

The largest of the immunoglobulins is IgM (Fig 13-15). It is a pentameric molecule consisting of five im-

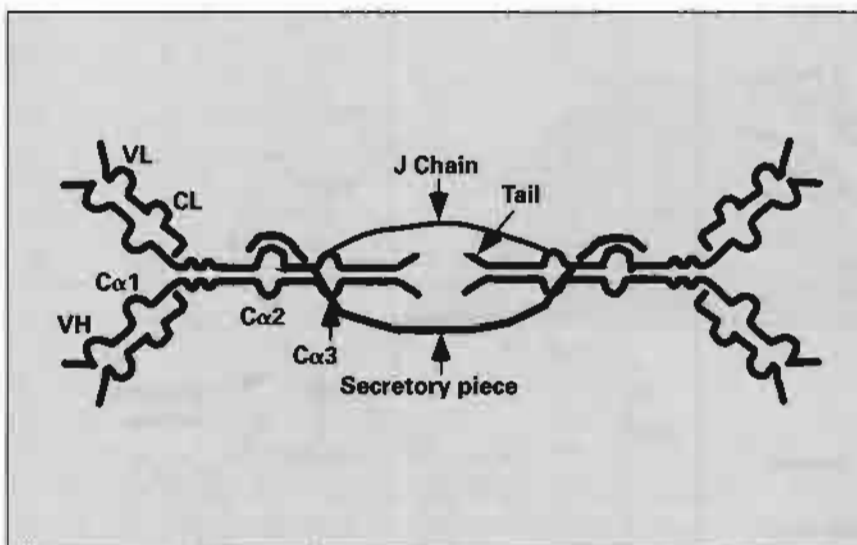


Fig 13-16 Structure of the immunoglobulin A secretory antibody. It consists of two immunoglobulin A molecules joined by a J chain polypeptide and a secretory piece, a protein added to IgA during its uptake and transport across epithelial cells. (CL) Constant light chain domain; (VH) variable heavy chain domain; (VL) variable light chain domain.

immunoglobulin units joined in such a way that their antigen-binding units radiate outward. The heavy chain polypeptides contain a fourth constant domain. The five heavy chains are joined near the carboxy terminals by disulfide bonds. A single cysteine-rich peptide, the J chain, links two of the immunoglobulins at their heavy chain by disulfide bonds to stabilize the radial structure (see Fig 13-15).

The IgA antibody molecule is constructed like the IgG molecule, in that it contains the variable light chain, constant light chain, variable heavy chain, and three constant domains on the heavy chains (Fig 13-16). In serum and extracellular fluid, the IgA molecule is a dimer of two immunoglobulins joined by a J chain. The dimeric IgA molecule is taken up by receptors on epithelial cells of intestinal mucosa and glandular acinar cells and transported into secretions as secretory IgA (see Fig 13-16).

The IgD and IgE antibody molecules, like IgG, are monomers consisting of two light chains and two heavy chains. They account for less than 2% of the antibody pool. The main differences between IgG, IgD, and IgE reside in the number and type of associated carbohydrate residues and in the number of nonvariable domains.

Secretory immunoglobulin A antibodies

Immunoglobulin A antibodies are produced in plasma cells located in the lamina propria of the intestinal and genitourinary tracts, bronchi, salivary

glands, and oral mucosa.¹²⁵ Numerous IgA-secreting plasma cells are located in the connective tissue of salivary glands, especially surrounding the excretory ducts of minor salivary glands. The IgA antibodies are directed against antigens that have penetrated epithelial barriers.

Soon after secretion from plasma cells, IgA antibodies are bound to receptors (polymeric Ig receptors) in the cell membrane of secretory epithelial cells, including salivary acinar and ductal cells. The IgA-receptor complex is taken up into the endosomal compartment and transported across the cell to the lumen, where it joins the secretions. The receptor molecule is retained as the secretory piece bound to the IgA dimeric molecules, forming a secretory IgA complex. The secretory piece protects the IgA antibodies from proteolytic cleavage in the extracellular secretions.¹²⁶

The major protective action of secretory IgA is its ability to bind to the surface of bacteria, thereby decreasing their attachment to epithelial cell membranes and their ability to colonize mucosal surfaces.

Keratinocytes of stratified squamous epithelia (including junctional epithelia) do not have polymeric Ig receptors and are unable to transport secretory IgA across mucosal epithelium. Immunoglobulin A secreted into the lamina propria connective tissue may cross junctional epithelium by diffusion through intercellular spaces, but not in the form of secretory IgA.

Lymphocyte Circulation from Bone Marrow to Secondary Lymphoid and Peripheral Tissues

The size of the circulating pool of lymphocytes is truly impressive: large enough so that an antigen will be bound and initiate a response within 2 hours of its gaining access to host tissues. To accomplish this, the entire pool of circulating lymphocytes must be replenished in approximately 2.5 hours. Naïve lymphocytes, differentiated to respond to a single antigenic peptide, migrate out of the thymus and the bone marrow and circulate to secondary lymphoid tissue via the bloodstream. In general, naïve T cells migrate via the bloodstream from bone marrow to the thymus, and subsequently to peripheral tissues and lymph nodes, by making contact with specific endothelial addressins.⁴ In contrast, naïve B cells travel directly from the bone marrow to lymph nodes, the spleen, Peyer's patches of the gut-associated lymphoid tissues, and the common mucosa-associated lymphoid tissues via the bloodstream. Both T and B cells exit the bloodstream through high endothelial venules located in lymphoid organs or in the common mucosal system (gut-associated, mucosa-associated, and duct-associated lymphoid tissues) in response to addressins and specific chemokines.^{5,127}

As the blood flows through the secondary lymphoid tissues, naïve and memory T and B lymphocytes migrate across HEVs to enter the extracellular spaces, where they interact with various types of dendritic cells.^{4,128} When these lymphocytes encounter specific (cognate) antigen, presented by APCs (dendritic cells) in the secondary lymphoid tissues, they begin a developmental process that includes clonal expansion of effector T cells, effector memory T cells, and high-affinity antibody (BCR)-bearing plasma cell precursors. These primed effector cells subsequently downregulate receptors for lymph node addressins and chemokines while up-regulating receptors for peripheral tissue and inflammatory chemokines and addressins.¹²⁹ Lymphocytes that fail to encounter specific antigen, presented by APCs in secondary lymphoid tissues, regain access to the bloodstream via lymphatics and the thoracic duct.

Effector lymphocytes settle in the lamina propria and/or epithelium of the oral and intestinal mucosa, the skin, certain glands, and in areas of inflammation by transmigrating across postcapillary venules. In

the lamina propria, effector lymphocytes function as helper T cells, cytotoxic T cells, and antibody-secreting plasma cells.¹²⁵ Exit from the postcapillary venules is regulated by the expression of various adhesion molecules (addressins) on endothelial cells as well as selectins and integrins on the circulating lymphocytes.^{4,128} Progress in this area suggests that different subsets of lymphocytes express "homing" receptors that allow them to be recruited to specific sites in the body by making contact with endothelial adhesion molecules (addressins).⁴

The preferential accumulation of T and B cells in peripheral tissues is due not only to endothelial cell interactions but also to local tissue factors that promote the survival of the lymphoid cells by inhibition of apoptosis. Without survival signals, 70% of lymphocytes die within 24 hours following activation.

Selective recirculation of lymphocytes to the initial site of antigen entry is suggested by the observed long-term presence of memory cells in mucosal tissue following a mucosal antigen challenge and in the systemic compartment (spleen, nodes, etc) when the initial exposure to antigen is systemic.¹³⁰ Intranasal immunization leads to increased long-term memory cells in the mucosa-associated lymphoid tissues but only a brief presence of memory cells in the components of the systemic compartment, for example, in the spleen. Of special interest is the finding that following an initial immunization of MALT, long-term memory cells spread through all parts of the common mucosal immune system.^{125,131} Thus intranasal immunization leads to recirculation of memory cells to other mucosal sites such as salivary glands and genital mucosal sites.¹³⁰

Lymphocyte-endothelial interactions during transmigration

Lymphocyte transmigration across blood vessels is regulated by the expression of specific cell surface adhesion molecules (addressins) in endothelial cells.^{4,132-134} The process involves several sequential receptor-ligand interactions involving lymphocyte and endothelial cell surface transmembrane glycoproteins, integrins, and chemokines.⁴ These interactions cause lymphocytes to roll slowly along the endothelial surface, inducing subsequent adhesion of lymphocytes to endothelium and increasing the activation of cytoskeletal elements needed for directed cell migration.

Because the flow of blood is slower in postcapillary venules, there is a greater chance for momentary

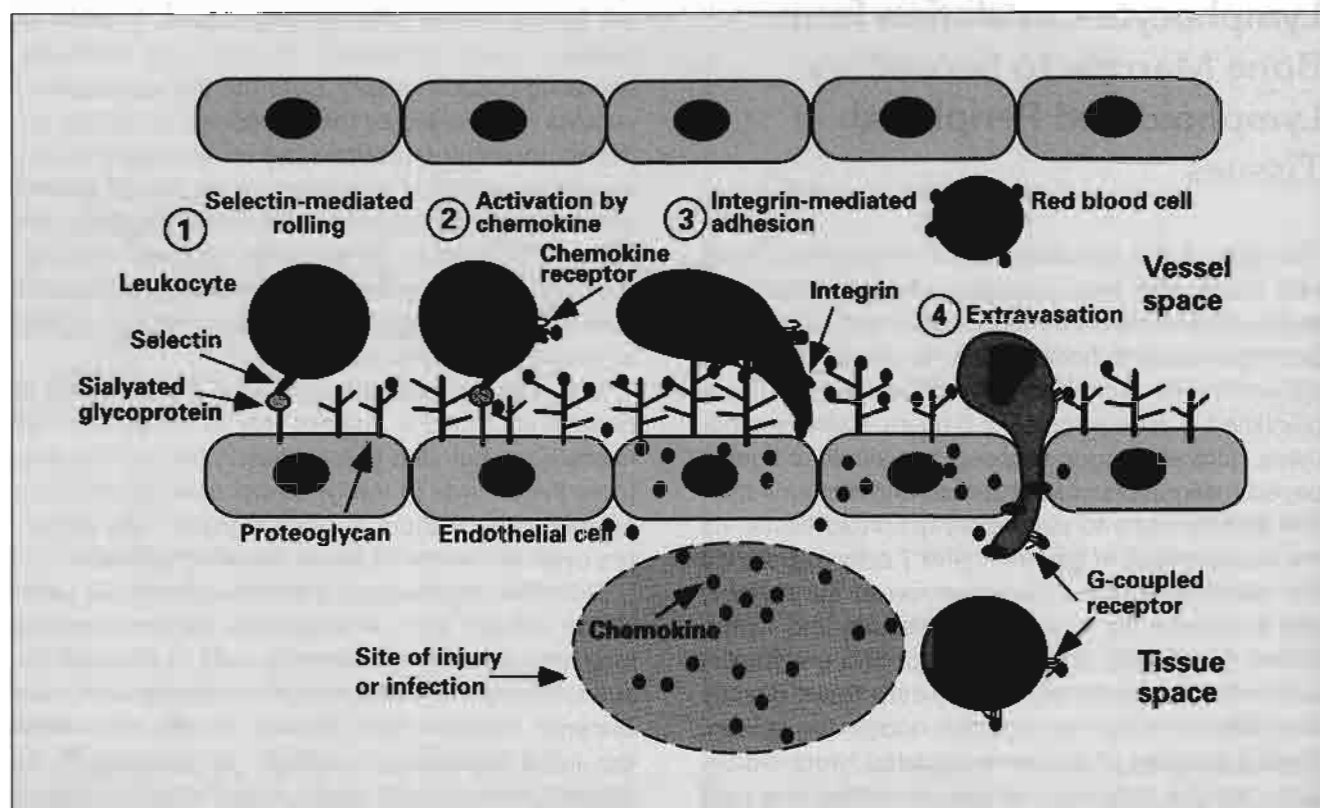


Fig 13-17 Steps in chemokine-induced movement of leukocytes from blood to the perivascular space. Weak adhesive interactions between selectins and their ligands on the endothelium lead to leukocyte rolling along the surface of the vascular wall. The binding of chemokines to plasma membrane G-coupled chemokine receptors triggers leukocyte activation with upregulation of integrins. Integrin-ligand interactions are responsible for directed cell migration between the endothelial cells and into the perivascular space. Chemokines are released in sites of inflammation by several cell types.

encounters between circulating leukocytes and endothelial cells. Low-avidity binding events between endothelial surface molecules occur, break, and reform, permitting a rolling-type contact during which the leukocytes appear tethered to the endothelium^{133,135} (Fig 13-17). Depending on the state of activation of the endothelial cell and the nature of the integrin adhesion receptors displayed on its surface, the leukocyte either will break free and reenter the flowing stream or will bind with increased avidity to the endothelium, and in the process undergo activation to a migratory phenotype. Integrin avidity and expression are rapidly upregulated by chemokine receptor signaling.

Leukocytes make initial (rolling) adhesive contacts between L selectins and their glycoprotein counterligands on the endothelium and between leukocyte glycoproteins and endothelial E and P selectins.^{4,133,135} Lymphocytes express L selectins that bind addressins (glycosylation-dependent cell adhe-

sion molecule 1 and CD34) on endothelial cells. Early adhesion during lymphocyte transmigration also involves the binding of integrin $\alpha 4 \beta 1$ (VLA-4) to its counter-receptor VCAM-1 and of cutaneous lymphocyte antigen to endothelial E selectin in dermal postcapillary venules.¹³⁶ In mucosal localization of lymphocytes, $\alpha 4 \beta 7$ binds to mucosal addressin cell adhesion molecule 1.^{10,137} The rolling adhesive contacts described are sufficient to permit lymphocyte chemokine receptors to engage respective chemokines that are bound on "activated" endothelial surfaces. Chemokine binding leads to lymphocyte activation.

Secondary binding, of higher avidity, occurs between additional integrins and their counterligands on the endothelium (LFA-1 to ICAM-1) following chemokine-induced activation of the lymphocyte. These secondary integrin-ligand interactions arrest the lymphocyte; more importantly, they participate in activating signaling cascades that stimulate the mi-

gratory process.^{133,138} Lymphocytes exit the bloodstream by migrating through the intercellular space between endothelial cells of postcapillary venules, especially the HEVs of organized lymphoid tissues (nodes, spleen, and Peyer's patches).

Interacting pairs of selectins, integrins, and cell adhesion molecules are shown in Fig 13-18. Lymphocyte CD44 binds endothelial hyaluronic acid. Both molecules are increased in inflammation, suggesting that CD44 binding to hyaluronic acid might promote lymphocyte extravasation in sites of inflammation.¹³⁹

Integrins are heterodimers of α and β subunits (see chapter 1). Each subunit is a transmembrane protein with an intracytoplasmic domain of potential importance in signaling pathways. Integrin-ligand interactions are involved in lymphocyte activation and migration.¹⁴⁰ Ligand binding to the extracellular receptor site can result in a conformational change in a subunit of the dimer and indirectly initiate a cytoplasmic phosphorylation event (an outside-in signal). In contrast, a change in the cytoplasmic domain caused by an interaction with another cytoplasmic protein may alter the external receptor site and change the avidity or degree of ligand binding (an inside-out signal). The outside-in mechanism, activated by the initial lymphocyte-endothelial interaction, triggers increased affinity between integrins and counterligands, leading to migration across the vessel wall.

Of the many mechanisms for recruiting lymphocytes to sites of inflammation and foreign antigen exposure, two are of crucial importance: the local elaboration of chemokines (and proinflammatory cytokines) and the expression of class I and class II MHC molecules on endothelial cells. Chemokine research is one of the fastest growing areas of immunologic and inflammation biology. At least 45 chemokines and 17 chemokine receptors have been identified.¹⁴¹

Specific chemokines and their receptors are now known to regulate the traffic of T_H1 and T_H2 cells, dendritic cells, eosinophils, neutrophils, and basophils into normal as well as inflamed tissue sites.^{70,141,142} Specific chemokines regulate naïve T- and B-cell traffic in secondary lymphoid tissues. Chemokines (see chapter 5), produced by a variety of cell types in response to injury, are bound on endothelial cell membrane proteoglycans (see Fig 13-17). Both T and B cells respond chemotactically to specific chemokines.^{6,143}

The concentration and display of chemokines on the endothelial surface provide an increased opportunity for binding to chemokine receptors on lymphocytes. Chemokine receptors are seven-pass

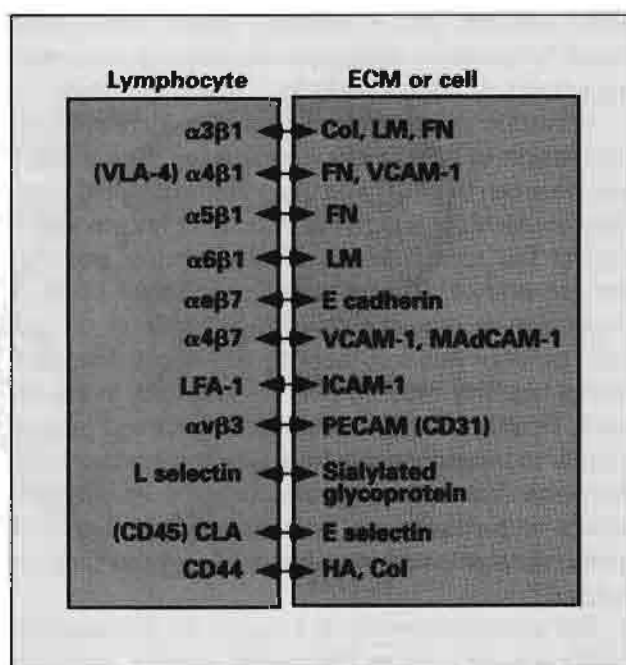


Fig 13-18 Partial list of lymphocyte plasma membrane molecules and their binding partners in the extracellular matrix (ECM) or on the surface of other cell types. (CLA) Cutaneous lymphocyte antigen; (Col) collagen; (FN) fibronectin; (HA) hyaluronic acid; (ICAM-1) intercellular adhesion molecule 1; (LFA-1) leukocyte function antigen 1; (LM) laminin; (MAdCAM-1) mucosal addressin cell adhesion molecule 1; (PECAM) platelet-endothelial cell adhesion molecule; (VCAM-1) vascular cell adhesion molecule 1. The $\beta1$ integrins are members of the very late activation (VLA) family of integrins.

transmembrane G protein-coupled receptors that initiate activation cascades in the lymphocytes.¹⁴⁴

A new class of CC chemokines specific for lymphocytes has been identified and cloned. These chemokines are believed to be involved in the trafficking and homing of lymphocytes to specific sites.^{5,144,145} One consequence of chemokine activation of lymphocytes is an increase in the avidity of integrin binding. Thus, endothelial cells indirectly participate in activating the migratory phenotype of lymphocytes by trapping chemokines. More than 25 different chemokines along with 12 specific chemokine receptors have been shown to regulate leukocyte behavior.^{6,70,127,141,144}

Chemokines, proinflammatory cytokines, and endotoxins also have an activating effect on endothelial cells, thereby increasing the expression of certain adhesion molecules such as ICAM-1 (CD54), which acts as counterligand for the $\beta2$ integrin (the CD18 component of LFA-1); VCAM-1, which binds to the

$\alpha 4\beta 7$ integrin; and E selectin, which binds cutaneous lymphocyte antigen, a mucinlike glycoprotein expressed on memory T cells.

Activation of endothelial cells also increases the expression of MHC class II molecules. The interaction between the TCR and the endothelial MHC complex could allow antigen-specific lymphocyte selection at the local site of infection and/or point of antigen entry.¹⁴⁶ The potential importance of MHC molecules in regulating the transmigration of specific lymphocytes into appropriate tissue compartments has only recently been recognized. In this regard, T-cell homing and retention in inflamed gingiva appear to be antigen directed. Gingival infection with *Actinobacillus actinomycetemcomitans* in rats with otherwise restricted oral flora induces retention of *A. actinomycetemcomitans*-specific T cells in gingival tissues.¹⁴⁷

The increased avidity of integrins for extracellular matrix ligands such as fibronectin, laminin, and type IV collagen initiates migration toward the basal lamina and ultimately promotes the retention of lymphocytes in areas of inflammation. The VLA subfamily of integrins is particularly significant in leukocyte migration in connective tissue.¹³³ Another consequence of the activation of the migratory state is the expression and secretion of matrix metalloproteinases capable of degrading the basal lamina, permitting the lymphocytes to breach that barrier and enter the adjacent connective tissue.

High endothelial venules

Naïve T and B lymphocytes exit the blood to enter the paracortex of lymph nodes through HEVs.¹⁴⁸ High endothelial venules are characterized by cuboidal endothelial cells that contain moderate amounts of endoplasmic reticulum and relatively large Golgi complexes.¹⁴⁹ These cells are specialized for constitutive expression of specific leukocyte adhesion molecules and chemokines. Peripheral node addressin, a carbohydrate-rich transmembrane protein that binds L selectin on leukocytes, is expressed on the HEVs of lymph nodes, while mucosal addressin cell adhesion molecule 1 is expressed on the HEV of Peyer's patches.^{4,127} The endothelial cells of HEVs also express ICAM-1, ICAM-2, VCAM, and E selectin (ELAM-1). High endothelial venules are also located in mucosal lymphoid tissues (adenoids and tonsils).

The segregation of T and B cells to distinct zones of lymph nodes is a good example of chemokine regulation of lymphocyte traffic. Peripheral naïve T lymphocytes express the chemokine receptor 7 (CCR7)

that binds CCL21, a chemokine expressed by endothelial cells of HEVs.¹²⁷ Formerly called *secondary lymphoid chemokine*, CCL21 triggers integrin-dependent adhesion of T cells to the endothelium. A similar, but as yet undefined, chemokine-chemokine receptor interaction regulates naïve B-cell binding to HEVs. Endothelial binding of B cells occurs along a different segment of the HEV, allowing B and T cells to segregate to different zones within the lymph node.

Following activation by interaction with APCs in the lymph node, lymphocytes downregulate chemokine receptors and adhesion molecules used in crossing HEVs and subsequently upregulate the expression of a different spectrum of chemokine receptors and adhesion molecules that are needed to direct their entry into peripheral tissues, especially in areas of inflammation. For example, CCR4, expressed on memory T cells, is activated by the chemokine CCL17 (thymus and activation-regulated chemokine), which is expressed on skin lamina propria endothelium, thereby directing T cells to skin.^{127,150} Signaling by thymus and activation-regulated chemokine increases the binding strength of the LFA-1-ICAM-1 interaction.

Lymph vessels and lymph nodes

The lymphatic system is a remarkable assembly of conduits, cells, and filtering stations designed to monitor the quality of the body fluids. Lymphatics arise in the connective tissues as blind-ended vessels. Lymphatic vessels collect up to 50% of the fluid and serum proteins that escape from capillaries and return them to the circulation after filtration in lymph nodes. The remaining 50% of the serum protein exudate returns to the circulation by reentering venules in the connective tissue. It is estimated that during a single day the entire volume of serum proteins escapes from the blood vessels and returns to the circulation via veins and lymphatic vessels.

Lymphatics also collect molecules that have gained access to the extracellular fluids from foreign sources or from the proteolytic degradation of matrix proteins. Antigens that gain access to lymphatics are drained into local lymph nodes, collected by APCs (follicular dendritic cells), and processed for presentation on MHC-II molecules. Peripheral lymphocytes and dendritic cells return to secondary lymphoid tissues by gaining access to local lymphatics. Antigen-activated dendritic cells also home to lymph nodes from peripheral tissues via afferent lymphatics.

Naïve T and B cells are activated in nodes on interaction with antigen-bearing APCs. Communication via lymphocyte-specific chemokines and chemokine

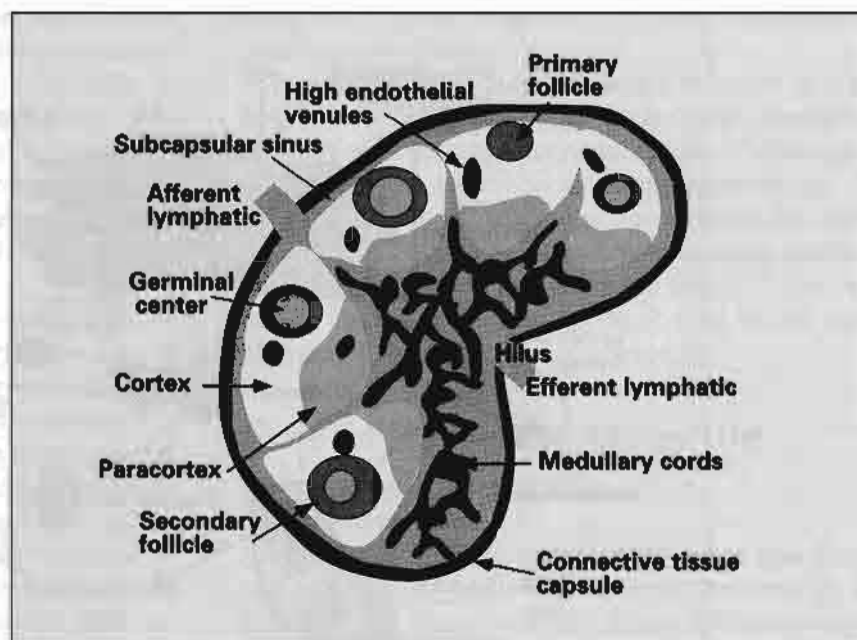


Fig 13-19 Typical lymph node. Lymph flows into the cortical spaces from afferent lymphatic vessels and drains into the medullary sinuses before joining the efferent lymphatic vessel at the hilus. The blood supply enters and leaves via the hilus. The cortical regions are mainly populated by B cells, while the paracortex is rich in T cells. Lymphocytes exit the bloodstream in high endothelial venules in the cortex and paracortex.

receptors has evolved to ensure that antigen-activated T and B cells encounter each other in the secondary lymphoid tissues, thereby mounting an efficient immune response.^{4-6,144} Activated lymphocytes also circulate to peripheral tissues for reactivation as effector cells in antigen-containing sites. It is not clear if naïve lymphocytes can be primed in peripheral tissue or whether initial priming must occur in secondary lymphoid tissues.

Lymph nodes are strategically located to filter antigens from incoming lymphatic fluid and to provide a stromal supporting network for the interaction of lymphocytes, macrophages, and APCs. Afferent lymphatic vessels merge with the subcapsular sinus, emptying lymphatic fluid into a complex system of sinusoidal spaces (Fig 13-19). Lymph flows sequentially from the subcapsular sinus through cortical, subcortical, and medullary sinuses before exiting the node through an efferent lymphatic vessel.

The sinuses are lined partially by endothelial cells, which are supported by reticular fibers. There is no basal lamina to separate the sinusoidal content from the stromal extracellular spaces. The stromal connective tissue of the cortex and paracortex consists of an open network of reticular fibers backed by larger collagen bundles.

The stromal connective tissue elements become more condensed as they converge to form medullary cords located between the medullary sinuses (see Fig 13-19). The APCs of the stroma consist of follicular

dendritic cells, located mainly in the cortex, and interdigitating dendritic cells, found mostly in the paracortex. Macrophages and plasma cells are present in high numbers within the medullary cords and sinuses.

Lymphocytes are concentrated in the cortex and paracortex of the node. They form follicular bodies during lymphocyte activation and proliferation. Germinal centers within secondary follicles represent sites of B-cell differentiation.^{92,125}

Mucosa-associated lymphoid tissue

Lymphoid cells are distributed throughout the mucosal tissues, forming a common and essential component of the body's surveillance system.¹⁵¹ The B cells sensitized in one part of the mucosal system are redistributed as antibody-secreting cells to all parts of the MALT following clonal proliferation and maturation in germinal centers of various nodal sites (Peyer's patches, tonsils, etc).¹²⁵ Within MALT, dendritic cells along with T and B cells provide a surveillance and reactive system designed to combat foreign antigens before they can gain access to the interior milieu of the body.

The CD8⁺ lymphocytes are located mainly within the epithelium. Intraepithelial lymphocytes are mostly CD8⁺ cytotoxic cells of the $\gamma\delta$ TCR type. They exhibit a variety of cytotoxic activities, including the destruction of pathogen-infected epithelial cells and the secretion of chemokines. The intraepithelial lymph-

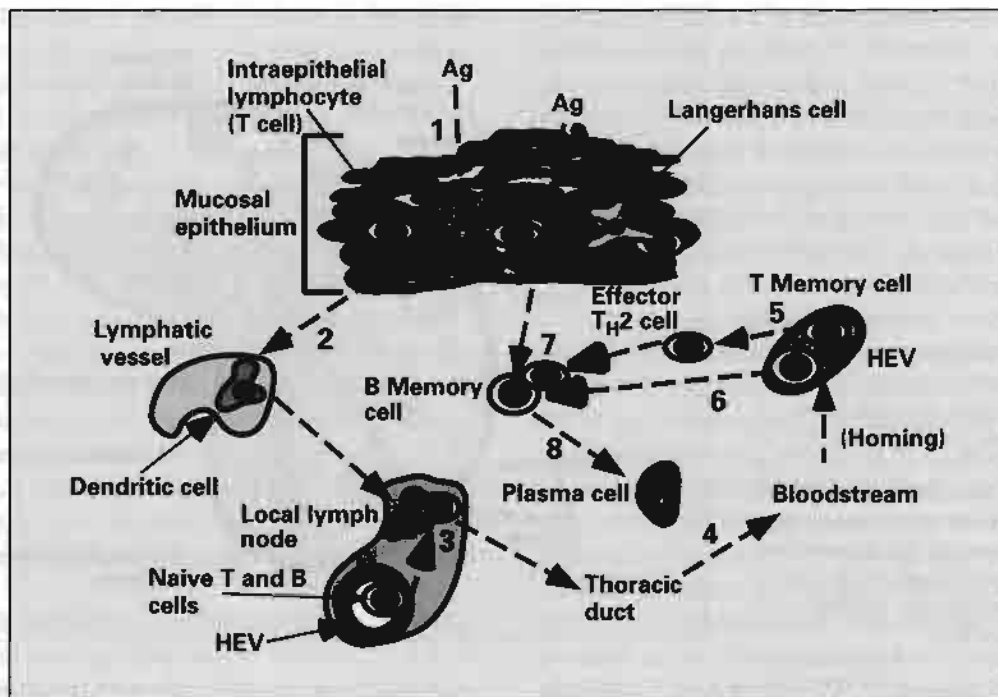


Fig 13-20 Lymphocyte traffic in mucosal tissues. (1) Antigens (Ag) that penetrate the epithelial layers contact and activate Langerhans cells, triggering their migration via lymphatic vessels to (2) local lymph nodes, where they assume a follicular dendritic shape. Antigens may also enter the lymphatic fluid and be transported to the lymph node. (3) Naive T and B cells enter the nodes via high endothelial venules (HEV) and interact with antigen-presenting cells (follicular dendritic cells and interdigitating cells). (4) After activation and clonal expansion, effector T and B cells leave the nodes via the efferent lymph and gain access to the bloodstream through the thoracic duct. (5 and 6) Memory T and B cells exit the blood through high-endothelial-like venules in the lamina propria. (7 and 8) Following binding and processing of specific antigen, B cells interact with helper T (T_H2) cells prior to differentiating into plasma cells. Effector T_H1 cells and $CD8^+$ cytotoxic T cells are similarly activated in secondary lymphoid tissue and migrate to the lamina propria site of antigen entry.

phocyte pool functions as a first line of defense, through cytokines, B-cell activation, and cytotoxic interactions following activation.^{78,152}

In contrast, $CD4^+$ lymphocytes are found mostly in the lamina propria of most mucosal surfaces and in large nodal aggregates in the tonsils, adenoids, and intestinal Peyer's patches. Lymphocytes of the mucosal lamina propria are predominantly activated T_H cells, memory B cells, and IgA-secreting plasma cells. Activated B cells play a significant role as antigen-presenting cells in their interaction with T_H2 cells in the mucosal lamina propria.¹²⁵ In inflammatory lesions of the lamina propria, there are also IgG- and IgM-secreting plasma cells.

The most extensive component of the MALT system is located in the intestinal mucosa: the gut-assoc-

ated lymphoid tissue. Intestinal epithelial cells express MHC-I and MHC-II molecules. Antigen presentation by intestinal epithelial cells to intraepithelial T cells ($CD8^+$) occurs via MHC-I interaction. Presentation of antigen on MHC-II occurs on intestinal epithelial cell processes that penetrate the basal lamina to contact $CD4^+$ T cells in the lamina propria.¹⁵³ Whether or not antigen presentation by intestinal epithelial cells leads to productive immunity or tolerance appears to be a function of appropriate costimulation. The presence of inflammation increases the level of intestinal epithelial cell-T-cell costimulation.

Epithelial cells of the gut (enterocytes) express MHC-I and MHC-II molecules and process and present antigens to intraepithelial lymphocytes.¹⁶³ Activated enterocytes¹⁶⁴ are capable of generating high

levels of costimulation and thereby activate T cells locally. Oral tolerance is induced by antigen-specific activation of suppressor CD4 and CD8 cells in Peyer's patches.¹¹⁷ Following induction, these cells migrate to other parts of the common mucosal system. After reencounter with the activating antigen, they produce and release large amounts of TGF- β , a potent immunosuppressive cytokine.¹¹⁷ This process is known as *bystander suppression*.

Naïve T and B cells migrate to lymph nodes via high endothelial venules (Fig 13-20). Naïve T cells may also return to lymph nodes from connective tissue via draining lymphatic vessels in the lamina propria. After migration to a local lymph node, naïve T and B cells may become activated following interaction with APCs bearing specific antigens and subsequently undergo proliferation and maturation as effector cells before returning to the bloodstream via the thoracic duct (see Fig 13-20). Dendritic cells, activated by antigen capture, migrate to local lymph nodes where they present antigenic peptides to naïve T cells. In a complicated homing process regulated by cell surface adhesion molecules on lymphocytes and endothelial cells, primed lymphocytes reenter the mucosal lamina propria via postcapillary venules that share many of the same characteristics as high endothelial venules.

It was recognized early on that epidermis, dermis, and regional lymph nodes constitute a skin-associated lymphoid tissue.¹⁵⁴ All the components needed to mount an immune response, ie, Langerhans cells, dendritic cells, T cells, and appropriate homing signals, have been demonstrated to exist in skin-associated lymphoid tissue. The key role of activated keratinocytes is described in the next section. The mucous membranes of the mouth are protected by a similar system. Within hours of antigenic challenge, CD4⁺ and CD8⁺ T cells invade the lamina propria and epithelium, along with an infiltration of neutrophils and MHC-II-positive dendritic cells and macrophages.¹⁵⁵ Plasma cells begin to appear in the affected site 1 week later.

An analogous lymphoid system has been identified within glandular tissues, such as the salivary glands, where lymphoid cell concentrations occur around the excretory ducts—the so-called duct-associated lymphoid tissue.¹⁵⁶ The production of IgA in duct-associated lymphoid tissues and its secretion as secretory IgA plays a major role in protecting the oral mucosa from viral and bacterial organisms. Recent evidence suggests that nasal lymphoid tissues (adenoids and tonsils) may have a prominent role in activation and regionalized distribution

of B cells to salivary (duct-associated lymphoid tissue) glands.¹⁵⁷

In addition to immune activation, the cells of the MALT also play a key role in immune tolerance through negative regulatory interactions.¹³⁷ Although mucosal (oral) tolerance is poorly understood, T cells are known to become antigen-tolerant by antigen-MHC-TCR binding without simultaneous costimulation in the lamina propria of the MALT.¹²⁵ The recently discovered T_H3 suppressor cells of the gut also play a key role in oral tolerance.¹²¹

Role of Keratinocytes in the Immune Response

Because oral mucous membranes come into contact with myriad food and microbial antigens on a regular basis, there must be a safety mechanism for minimizing harmful inflammatory reactions while maintaining the ability to mount a defensive response to pathogens. This is accomplished in a precisely coordinated system involving surveillance, activation, and suppression. Although many of the details of this system have yet to be defined, it should be apparent that this area represents a fertile field with great significance to oral health and disease.

It has often been suggested that the keratinocyte represents the first line of defense in the immune system of skin and mucosal surfaces. There is ample evidence that keratinocytes participate in regulating several facets of the local immune response by releasing cytokines.^{158–160} Keratinocytes express a limited number of cytokines constitutively. When injured, however, they have the ability to produce additional regulatory molecules that are released into the local environment (Fig 13-21). Approximately 20 different cytokines are produced by keratinocytes in response to various stimuli. Cytokines diffuse into the lamina propria to act on endothelial cells, fibroblasts, and macrophages. They also generate chemoattractant gradients for leukocyte infiltration.

Unstimulated healthy keratinocytes express IL-1 and its receptor (IL-1R). Small amounts of IL-6 and granulocyte-macrophage colony-stimulating factor (GM-CSF) are also produced. These cytokines are released only on cellular injury. Because keratinocytes express IL-1R, they can react to the release of IL-1 via both autocrine and paracrine pathways. The regulation of the number of IL-1Rs is an important mechanism for setting the level of keratinocyte response to injurious stimuli. For example, the expression of IL-6

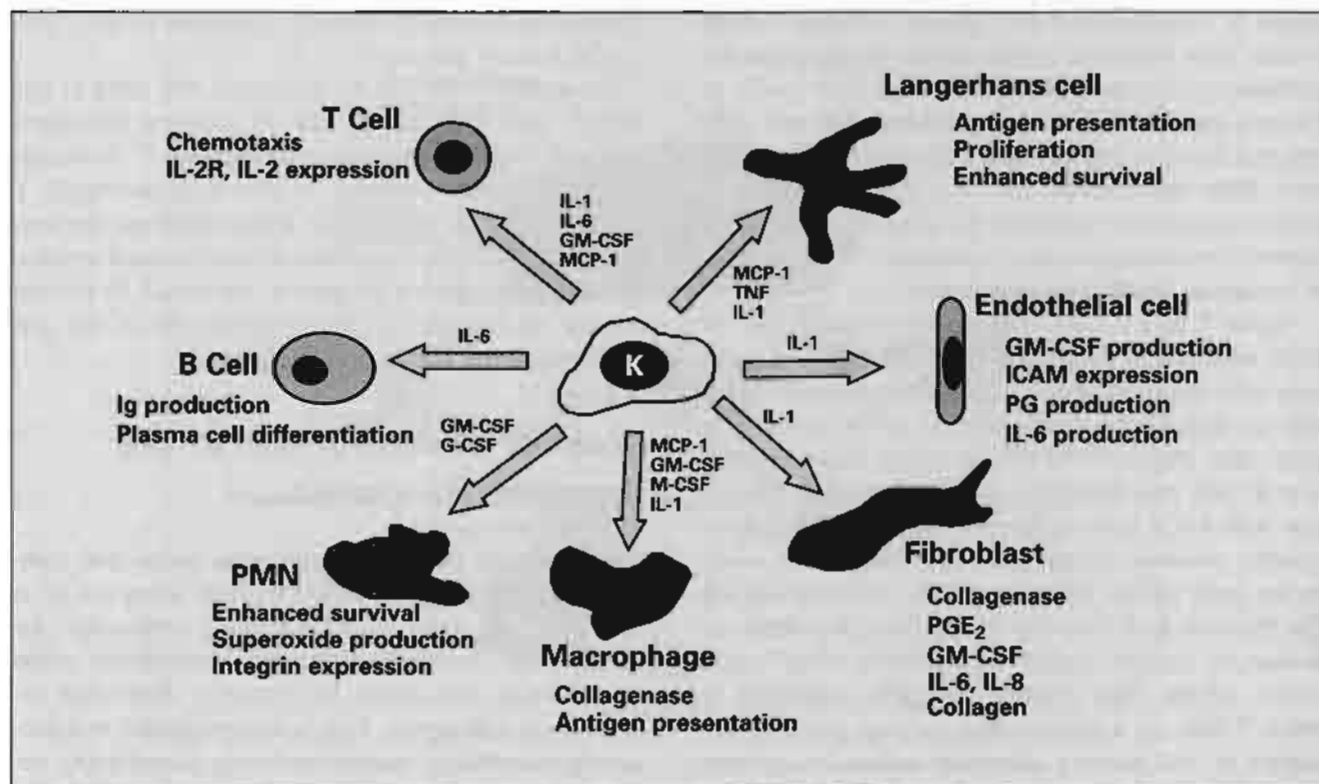


Fig 13-21 Potential regulatory functions of keratinocytes (K) via the production of various cytokines during inflammation. Cells of the immune system as well as connective tissue cells are target cells for interleukins 1 (IL-1) and 6 (IL-6). Macrophage chemoattractant protein 1 (MCP-1) and several colony-stimulating factors activate various leukocytes and antigen-presenting cells. Fibroblasts and endothelial cells respond to IL-1 by generating IL-8, which is a potent neutrophil chemoattractant. (G-CSF) Granulocyte colony-stimulating factor; (GM-CSF) granulocyte-macrophage colony-stimulating factor; (ICAM) intercellular adhesion molecule; (Ig) immunoglobulin; (IL-2R) interleukin 2 receptor; (M-CSF) macrophage colony-stimulating factor; (PG) prostaglandin; (PGE₂) prostaglandin E₂; (PMN) polymorphonuclear neutrophil; (TNF) tumor necrosis factor. (Adapted with permission from Kupper.¹⁶⁰)

and GM-CSF by keratinocytes is directly correlated to the level of IL-1R expression.

The release of IL-1, IL-6, TNF, and GM-CSF from injured keratinocytes has a potentiating action on several cell types located both within the epidermis and in the underlying lamina propria (see Fig 13-21). Langerhans cells are stimulated by IL-1 and GM-CSF to proliferate and to increase their capacity for antigen processing and presentation to intraepithelial lymphocytes. Intraepithelial memory T cells activated by the combined effect of keratinocyte cytokines and interaction with Langerhans cells generate IL-2, an essential factor for the differentiation of effector T cells, and IL-6, essential for plasma cell maturation.

Endothelial cells, fibroblasts, macrophages, and neutrophils of the lamina propria react to IL-1 and

GM-CSF by upregulating their level of activity (see Fig 13-21). Increased expression of ICAM-1 and LFA-3 on endothelial cells in response to IL-1 stimulates the transmigration of neutrophils and lymphocytes into the zone of injury. Endothelial cells express class II MHC molecules when activated by proinflammatory cytokines and endotoxins.

Another important consequence of the release of IL-1 is its action to increase the production of IL-6 by several cell types, including fibroblasts, endothelial cells, keratinocytes, and macrophages. Interleukin 6 potentiates the development of B cells and plasma cells and increases development of osteoclasts.

Keratinocytes can regulate T_H cell polarization in the epidermis and underlying dermis by varying the secretion of IL-10 and IL-12.¹⁶¹ In addition, they pro-

duce chemokines such as IL-8, a chemoattractant for neutrophils, and monocyte chemoattractant protein 1, a potent stimulator of monocyte-macrophage chemotaxis. Keratinocytes express CD40 in increased amounts when stimulated by IFN- γ . Engagement of CD40 by its ligand, a TNF-like soluble protein, activates secretion of IL-8 by keratinocytes.¹⁶²

Williams and Kupper¹⁶¹ have written an in-depth review of the role of the keratinocyte in regulating epidermal immune responses and inflammation.

Keratinocytes have been shown to respond to IL-4 by expressing MHC-II molecules, suggesting the possibility that activated keratinocytes might be able to act as APCs. The ability of the keratinocyte to secrete cytokines and to express MHC-II molecules has led to the notion that the epidermis might provide a thymus-like environment for the development of certain T-cell subsets. There is also a growing body of evidence that epidermal keratinocytes are involved in lymphocyte activation. The bulk of this evidence was obtained by studying epidermal cells, chiefly with *in vitro* culture systems. If similar functions can be demonstrated *in vivo* for oral keratinocytes, it would go a long way to increasing the understanding of oral mucosal immunity.

Role of Dendritic Cells and Langerhans Cells

Dendritic cells are specialized for capture and presentation of antigen to T and B cells. Immature dendritic cells, produced in bone marrow, circulate to peripheral tissues where they exit postcapillary venules following interaction with specific endothelial addressins and chemokines. Langerhans cells, a subset of the dendritic cells, leave dermal connective tissues to reside in the epithelium of skin and oral mucosa. Peripheral dendritic cells, including Langerhans cells, are differentiated for antigen capture.¹⁶⁵ They express numerous Fc receptors, Toll-like receptors, and pattern recognition receptors, making them well adapted for phagocytosis of microbes and particulate material.

Following antigen capture, dendritic cells undergo activation involving upregulation of MHC-II molecules, costimulatory proteins such as B7, and cytokines (especially IL-12). Activated dendritic cells migrate from peripheral tissues to secondary lymphoid tissues, serving as carriers of antigen for interaction with lymphocytes (see Fig 13-20). Activated dendritic cells remain viable for several days within secondary lymphoid tissues, during which time they activate T and B cells.

Activated dendritic cells express numerous plasma membrane proteins used in chemokine signaling, antigen presentation, and lymphocyte costimulation. Such molecules include CXCR5 and CCR7, chemokine receptors regulating entry into and within lymph nodes; CD1, a marker common to cells of the macrophage-dendritic cell line; CD14, a lipopolysaccharide-binding protein; CD29, a β integrin; CD45, the common leukocyte antigen; the MHC-II antigen receptor; receptors for the C3b component of complement and the Fc chain of antibody molecules; and several pattern recognition receptors, including the mannose receptor, and Toll family proteins.^{2,47,48,166,167}

Another important function of the dendritic cells and Langerhans cells, as sentinel cells, is their ability to secrete inflammatory chemokines that contribute to the recruitment of effector cells such as macrophages, granulocytes, and additional dendritic cells to early inflammatory lesions.¹⁶⁷

Langerhans cells are located just above the basal cell layer of the epidermis and the stratified squamous epithelium of most mucosal surfaces.^{47,48,168} Langerhans cells act as the first line of surveillance against foreign antigens. Immature Langerhans cells, of bone marrow origin, populate epithelial surfaces via the bloodstream. This physiologic migration of Langerhans cells through connective tissue is thought to be a response to chemotactic gradients originating from the epithelium. Monocyte chemoattractant protein 1 and GM-CSF have been implicated as potential chemoattractants.

Langerhans cells are found in all oral mucosal surfaces except the junctional epithelium and the base of the taste buds.¹⁶⁸ Buccal mucosa contains more than twice the number of Langerhans cells found in the hard palate and gingiva. They have a tendency to concentrate over dermal papillae, where the epithelium is thinner. Oral mucosal Langerhans cells share many of the same characteristics of epidermal Langerhans cells, including the expression of high levels of MHC-II molecules and the capacity to initiate costimulatory signals and cytokine secretion.¹⁶⁹ Dendritic cells are also found in the lamina propria of the oral mucosa, where they have a role similar to that of dermal dendritic cells.

Dermal dendritic cells are concentrated in perivascular sites. Langerhans cells and dermal dendritic cells probably represent two differentiation pathways from a more naïve blood-borne dendritic cell.¹⁷⁰

The epithelial Langerhans cell is characterized by a large surface area provided by numerous cytoplasmic processes extended between keratinocytes.¹⁷¹ Typi-

cally the dendritic cell processes are oriented toward the surface of the epithelium. Adhesion between Langerhans cells and keratinocytes is mediated by E cadherins. Unlike its neighboring keratinocytes, the Langerhans cell contains no keratin filaments and consequently it appears as a less dense and less intensely stained cell. Racket-shaped Birbeck granules, visualized by electron microscopy, are specific morphologic markers of the Langerhans cell. These granules are part of the endosomal system.

On antigen capture, including the internalization of bacteria, the Langerhans cell undergoes activation, causing it to migrate out of the epithelium and into a lymphatic vessel of the lamina propria^{172,173} (see Fig 13-20). It has been suggested that Langerhans and other dendritic cells may harbor viable bacteria for several hours and in some cases might carry the infection into deeper tissues.¹⁶⁵

In the migratory phase of the Langerhans cell, there is a decrease in the expression of E cadherins and an increase in the expression of integrins. These changes loosen attachments to epithelial cells while promoting attachments to components of the connective tissue matrix. Cytoskeletal changes needed in Langerhans cells for migration are stimulated by TNF- α and IL-1 β , cytokines produced by activated keratinocytes¹⁷⁴ (see Fig 13-21). During migration the Langerhans cell assumes a less dendritic shape, appearing more like a large, motile macrophage. An additional consequence of activation is increased expression of MHC-II and T-cell costimulatory molecules such as B7 (CD80).⁶⁶

In the environment of the lymph node, the dendritic shape returns as the Langerhans cell takes on the characteristics of the interdigitating antigen-presenting cell. Naïve T cells (mainly CD4⁺) are bound to the interdigitating antigen-presenting cell and undergo activation involving the MHC-II-TCR binding axis and the various cosignaling adhesive contacts (see Figs 13-6 and 13-7). The potency of the Langerhans cell in generating costimulatory signals is related to its level of B7 expression, a condition stimulated by IL-1 and GM-CSF.¹⁷⁵

Activated effector T cells, produced during interaction with interdigitating antigen-presenting cells, migrate from the lymph nodes via the efferent lymph, lymphatic duct, and bloodstream back to the peripheral site of antigen challenge. During this homing response, the T cells are guided by selectin, addressin, chemokine, and integrin recognition events (see Fig 13-20). After exiting from postcapillary venules, long-lived effector memory T cells and short-lived effector T cells populate the lamina propria and the epithelium.⁴

Basic Science Correlations

Signal transduction in lymphocyte activation

Antigen binding in T cells at the TCR, or in B cells at the BCR, starts a sequence of signaling events linked to the activation of numerous cytoplasmic enzymes whose products ultimately diffuse into the nucleus to regulate gene transcription.^{104,176,177} The complexity of the process is demonstrated by the fact that antigen binding rapidly induces transcription of approximately 30 separate genes. Furthermore, there is evidence that subsets of T lymphocytes demonstrate differences in signal transduction initiated from the TCR complex.¹⁷⁸

Basically, during lymphocyte activation, two independent signaling cascades become activated: The first pathway proceeds from the activation of phospholipase C γ , while the second pathway stems from the activation of a guanine nucleotide binding protein, p21^{ras}, and the mitogen-activated protein kinase (MAPK) cascade^{177,179-181} (Fig 13-22). Both pathways require initial phosphorylation of Src family kinases and protein tyrosine kinases.

The immunoglobulin molecules that make up the TCR and the BCR have short cytoplasmic tails and thus are incapable of effecting a signal transduction without the aid of additional associated transmembrane proteins. The CD3 complex of T cells and the Ig- α and Ig- β dimers of B cells connect the antigen recognition units with cytoplasmic signaling pathways.^{176,182} Each of the five polypeptides of the CD3 complex (and the Ig- α -Ig- β dimers of B cells) contain special tyrosine phosphorylation sites known as *immunoreceptor tyrosine-based activation motifs* (ITAMs)^{176,183,184} (see Fig 13-22). Each ITAM is made up of a short stretch of amino acids containing two tyrosine residues.

Immediately following antigen binding, members of the Src homology (SH) family of protein tyrosine kinases rapidly phosphorylate ITAM tyrosines. Phosphorylated ITAMs provide sites for the recruitment and activation of proteins that contain SH2 domains, such as protein tyrosine kinases and phospholipase C γ .^{179,184,185} (see Fig 13-22). First identified in Src proteins, SH domains contain short amino acid sequences that recognize and attach to phosphorylated tyrosines. Specificity of the binding interaction between SH domains and phosphorylated tyrosine is coded by a sequence of three amino acids positioned next to the tyrosine.

It appears that the CD4 (and CD8) coreceptor molecules of T cells are involved in binding and posi-

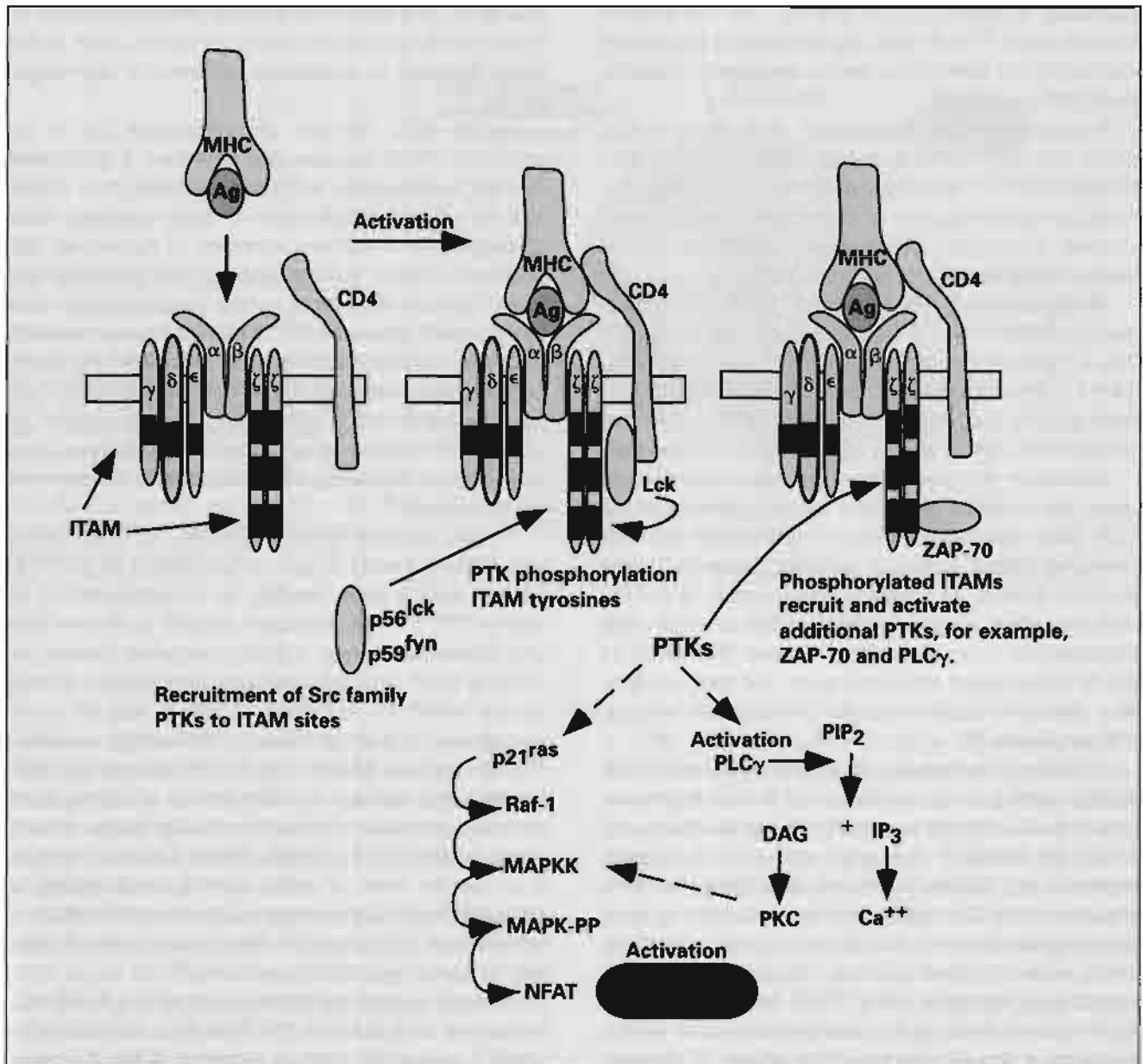


Fig 13-22 Signaling events following the binding of antigen (Ag) at the T-cell receptor involves phosphorylation of immunoreceptor tyrosine-based activation motif (ITAM) subunits of the CD3 proteins by p56^{lck}, a member of the Src family of protein tyrosine kinases. CD4 binds p56^{lck}, serving to position it close to the CD3 transmembrane proteins following CD4 binding to the major histocompatibility complex type I (MHC-I) molecule. Phosphorylated tyrosine residues of the ITAM subunits recruit and activate additional protein tyrosine kinases (PTKs) that lead to activation of the Ras-mitogen-activated protein kinase (MAPK) pathway. Mitogen-activated protein kinase phosphorylates nuclear factor of activated T cells (NFAT), the primary regulator of the interleukin 2 (*IL2*) gene. Activation of the phospholipase C γ (PLC γ) pathway for the generation of diacylglycerol (DAG) and inositol triphosphate (IP₃) from phosphatidylinositol-4, 5-bisphosphate (PIP₂) is triggered by the association of the PLC γ with phosphorylated tyrosine residues of ITAM subunits. (ZAP-70) Zeta-associated protein (70-kDa); (MAPKK) mitogen-activated protein kinase kinase; (MAPK-PP) mitogen-activated protein kinase, double phosphorylated; (PKC) protein kinase C. (Adapted in part from DeFranco¹⁸¹ with permission from Elsevier Science.)

tioning Src family proteins in close relationship to the ITAM units of CD3 molecules.¹⁸⁶ The association of CD4 (CD8) with the appropriate MHC molecule ensures the close approximation of Src and ITAM units

once a TCR-antigen-MHC bridge has formed (see Fig 13-22). It has been suggested that SH adapter proteins are already bound to CD4 before the antigen recognition event. Association between the ex-

tracellular domains of CD4 and the TCR enhance T-cell activation.¹⁸⁷ In B cells, the coreceptor function is believed to be carried out by the complement receptor (CR2) and CD19.

Following antigen stimulation, the Src proteins p56^{lck} and p59^{fyn} phosphorylate ITAMs in T cells and B cells.^{59,184,185,188} Deletion of p56^{lck} in mice results in almost complete absence of thymic lymphocyte development. In contrast, a high level of p56^{lck} expression leads to development of aggressive thymic tumors.¹⁸⁹

Phosphorylated ITAM units of CD3 recruit and phosphorylate 70-kDa zeta-associated protein (ZAP-70), a cytosolic protein kinase.^{62,177,183} Subsequently, ZAP-70 phosphorylates downstream substrates, leading to lymphocyte activation.¹⁷⁸ Deletion and/or mutation of ZAP-70 lead to serious T-cell deficiencies.

Activation of coreceptors that potentiate or suppress the stimulus generated by engagement of the TCR (and BCR) also involves sequential tyrosine phosphorylation events. A recently discovered cytoplasmic domain on inhibitory coreceptors is the immunoreceptor tyrosine-based inhibition motif first identified on Fcγ receptors of B cells. Activation of the tyrosine-based inhibition motif unit through tyrosine phosphorylation recruits SH2-domain-bearing phosphatases.¹⁹⁰

Tyrosine phosphatases (the most highly studied is CD45) participate in regulation of T- and B-lymphocyte activation by dephosphorylating tyrosine kinases of the Src family.¹⁰⁴ Activation of T cells is severely impaired in CD45-deficient animals. Some tyrosine kinases of the Src family must be activated by prior dephosphorylation of a negative regulatory domain, while other tyrosine kinases may become deactivated by dephosphorylation.¹⁹¹⁻¹⁹³ Another key activity of CD45, through its dephosphorylation of Src proteins, is the regulation of the affinity of lymphocyte integrins for their counterligands.¹⁹³

Newly discovered proteins called *transmembrane adaptor proteins* assist the recruitment of cytoplasmic signaling proteins to the TCR complex.^{194,195} These adaptor proteins have short extracellular domains and relatively large cytoplasmic tails containing tyrosine-based activation motifs capable of interactions with SH2 domains of Src proteins and cytoplasmic tyrosine kinases. A newly discovered adaptor protein, T-cell receptor interacting molecule, is activated along with the TCR and engages in SH2-mediated signaling events.¹⁹⁴

Another accessory signaling receptor associated with the TCR is the CD2 glycoprotein. Activation of CD2 results in tyrosine phosphorylation events similar to those following CD3 engagement. Downstream

events of CD2 activation include phosphorylation of fimbrin and dephosphorylation of cofilin, both molecules involved in regulating cytoskeletal rearrangements.¹⁹⁶

Via its SH2 domain, phospholipase Cγ is recruited to ITAM subunits and activated. It generates inositol triphosphate and diacylglycerol from cleavage of phosphatidylinositol-4, 5-bisphosphate (see chapter 9). In a pathway common to numerous signaling molecules, inositol triphosphate liberates Ca²⁺ from intracellular stores, while diacylglycerol activates protein kinase C.^{177,197} It has also been reported that the phosphatidylinositol 3-kinase signaling pathway is activated during B-cell differentiation, especially important in stimulating the expression of CD40.¹⁹⁸ Rho family guanosine triphosphatases play key roles as signaling intermediates in lymphocyte development.¹⁹⁹

Protein tyrosine kinases activated by association with ITAM subunits trigger the activation of p21^{ras} in both T and B cells, leading to phosphorylation of MAPK.^{62,200-202} This has been shown to involve several downstream steps requiring separate kinases, including Raf-1 and mitogen-activated protein kinase (MAPKK). Activation of MAPK follows phosphorylation of both tyrosine and threonine residues. Phosphorylated MAPK (MAPK-PP) stimulates DNA transcription through the intervention of various transcription promoters, including nuclear factor of activated T cells (see Fig 13-22). Protein kinase C also influences the level of active MAPK via its ability to stimulate Raf-1. Superantigen activation of B cells has been shown to proceed via the phospholipase C, protein kinase C, and MAPK cascade.²⁰³

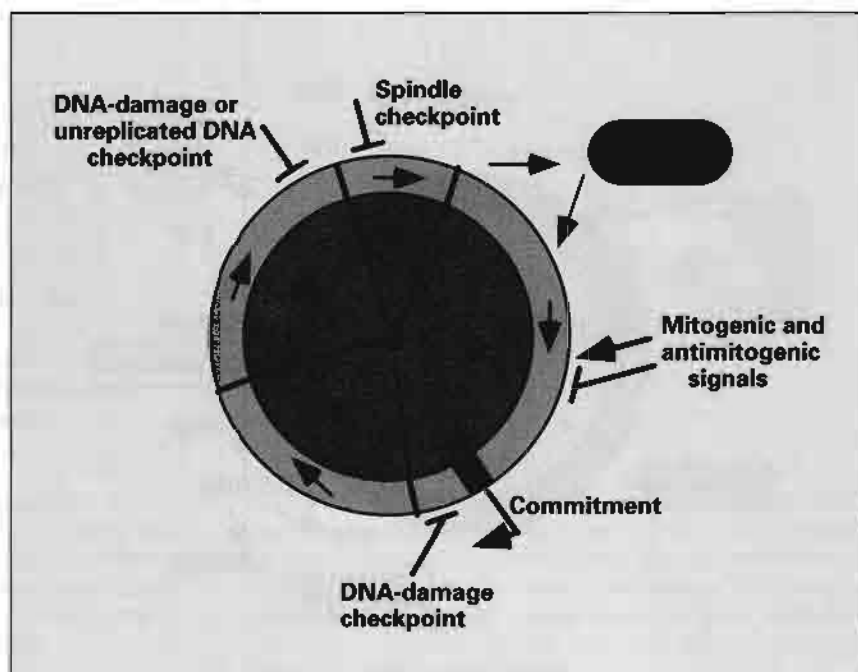
The prime gene transcripts induced by T-cell activation are for IL-2 and IL-2R.²⁰⁴ Nuclear factor of activated T cells is the primary regulator of the IL2 gene. Recent evidence supports CD28 activation of nuclear factor of activated T cells without simultaneous TCR signals.²⁰⁵ This observation supports the concept that certain T-cell functions, such as IL-2 production, may occur without specific antigen activation.

Intracellular signaling events following activation of T cells also lead to the phosphorylation of actin-binding proteins (fimbrin and cofilin), suggesting that cytoskeletal changes form part of the activating process.

Cell division

Repeated cell division is involved in generating the great diversity of antigen recognition sites during the differentiation of T and B cells. Rapidly proliferating

Fig 13-23 Four phases of the cell division cycle, indicating the positions of DNA-damage and spindle checkpoints, as well as the location of the restriction (R) point, beyond which the cell must divide or undergo programmed cell death. On completion of cell division, cells may undergo cell differentiation and enter the G_0 state. Mitogenic growth factors (and antimitogenic factors) act during G_1 , (G) Gap phases; (M) mitotic phase; (S) synthesis phase. (Adapted with permission from Grana and Reddy.²⁰⁸)



cell populations are susceptible to DNA replication damage and are the origin of many cancers. Thus it is appropriate at this point to review some of the basic mechanisms that control the cell division cycle and to point out certain defects in regulatory molecules that can lead to cancer.

Cell division involves the duplication of the cell's nuclear and cytoplasmic components, followed by their equal distribution into two daughter cells. The complexity of this process is enormous, yet it takes place successfully millions of times per day in the human body. Renewal of the skin and mucous membranes, the lining of the intestine, and the cells of the hematopoietic and immune systems requires rapid and accurate cell division. Once a cell has been stimulated to undergo division, the process occurs over a period of 10 to 20 hours.

The cell division cycle proceeds in four phases (Fig 13-23): a period of DNA synthesis, the S phase, during which the cell's genetic code is replicated; a phase of mitosis, or M phase, involving the equal distribution of chromosomes and cytoplasmic material to two daughter cells; and two time gaps, the G_1 and G_2 phases, during which synthetic and regulatory events take place to ensure that the S and M phases occur in an orderly and accurate manner.²⁰⁶

Following mitosis (and cytokinesis), the life span of a daughter cell begins with entry into the G_1 phase.

In cells programmed to undergo repeated cell division, the G_1 phase can be as short as 5 hours. However, many cell types are arrested in G_1 for relatively long periods of time and must be stimulated to proceed into cell division by growth factors or mitogens.

To progress from G_1 to the S phase, the cell must pass a restriction point (see Fig 13-23). Restriction is lifted once an appropriate level of regulatory factors, chiefly cyclins and cyclin-dependent kinases, has been reached in late G_1 . The S phase typically requires 6 to 7 hours. Following completion of DNA replication, a short G_2 phase is required to assemble the molecular systems needed for mitosis and cytokinesis.

The M phase, requiring about 1 hour, is the shortest of all four phases. Two DNA-damage checkpoints, one prior to DNA synthesis and a second just prior to mitosis, must be crossed successfully or the cycle is interrupted and the cell undergoes programmed cell death.^{206,207}

Many cell types undergo terminal differentiation and leave the cell cycle by entering a G_0 state (Fig 13-24). Some cell types remain permanently in G_0 , while other cell types can be stimulated to reenter the cell division cycle by growth factors.

Several families of regulatory proteins peak and then decline at various points in the cell cycle. The key regulators are the cyclins and cyclin-dependent

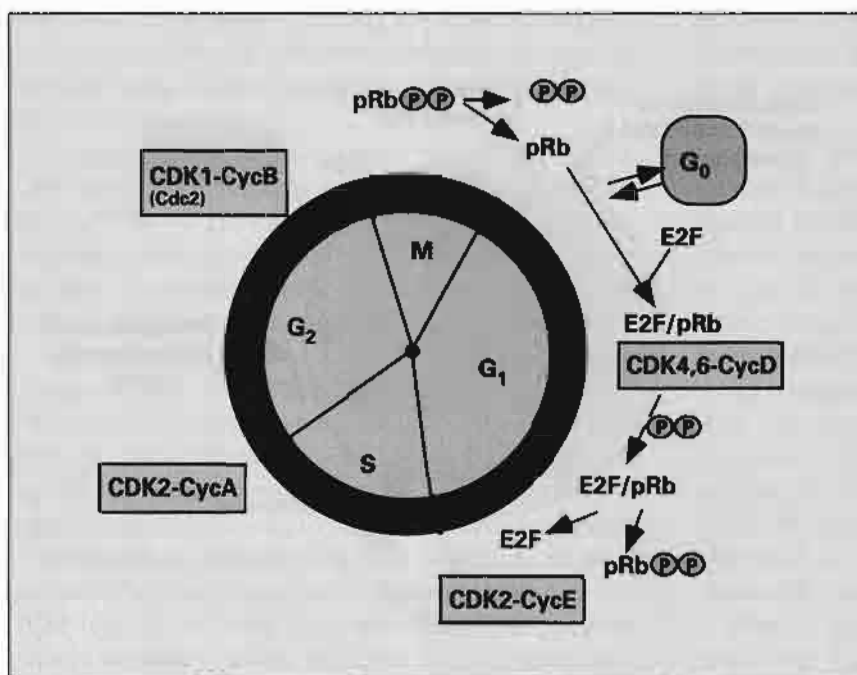


Fig 13-24 Points at which some of the major cyclin (Cyc)-cyclin-dependent kinase (CDK) complexes peak in relation to the phases of the cell cycle: (G) gap phases; (M) mitotic phase; (S) synthesis phase. The regulation of E2F via pRb is a key factor coupling growth factors (represented by epidermal growth factor) during G₁. The release of E2F transcription promoters is effected through the phosphorylation (P) of pRb by the complex CDK4,6-CycD. (Adapted with permission from Grana and Reddy.²⁰⁸)

serine-threonine protein kinases (CDKs).²⁰⁶⁻²⁰⁹ The cyclins are a family of regulatory proteins that associate with CDKs to regulate the timing, substrate specificity, and localization of CDK activity (Fig 13-25). Cyclin-CDK complexes are responsible for the phosphorylation of specific groups of proteins that drive the cell through the various phases of the cell cycle. Cyclin D, CDK4, and CDK6 are operative during G₁, driving cellular events toward the S phase (see Fig 13-24).

Activation of the cell cycle involves an increase in the availability of DNA regulatory proteins (transcription promoters) to start the task of DNA replication. A key pathway regulating DNA synthesis involves the family of E2F transcription promoters and E2F-binding proteins. The E2F proteins activate the process of DNA synthesis and replication.^{208,210,211}

In early G₁, E2F proteins are bound in an inactive form to members of another class of proteins, the tumor suppressor proteins. The prime member of this class is pRb, a protein discovered in human hereditary retinoblastomas.^{210,212,213} Cells of this tumor contain a mutated form of pRb that is incapable of binding E2F, thereby permitting unrestrained cell proliferation. The pRb-type tumor suppressor proteins are also called "pocket proteins" because they share homologous E2F binding domains, or pockets. The pRb protein must be in its hy-

pophosphorylated state to bind E2F. The phosphorylation of pRb by the cyclin D-CDK4 complex in G₁ releases E2F (see Fig 13-24).

Another cyclin-CDK complex is cyclin E and its binding partner CDK2. This complex peaks at the G₁-S junction. During the S phase, it phosphorylates other pRb-type pocket proteins (p107 and p130) that form inactivating complexes with members of the family of E2F transcription factors (Fig 13-26).

Cyclin A-CDK2 complexes peak during G₂ and the G₂-M junction. Cyclin B and Cdc2 (also known as CDK1) form complexes at the G₂-M transition point of the cycle. The substrates phosphorylated by cyclin A-CDK2 and cyclin B-Cdc2 have yet to be identified but are likely to involve elements of the cytoskeleton involved in chromosome transport and cytokinesis.

Negative regulation of the cell cycle occurs through the action of several small proteins, the cyclin-CDK inhibitors (CDKIs).^{206,208} These proteins either prolong the length of G₁ or completely inhibit cell division. They have been shown to increase in cells undergoing terminal differentiation. A member of the CDKI family, p21, inhibits the cyclin D-CDK4 complex at the G₁-S transition.²¹⁴ In addition, CDK4 and CDK2 are inhibited by both CDKI p27 and CDKI p16. Mutations in CDK inhibitors may convert them into oncogenes, leading to unregulated cell proliferation.^{206,207}

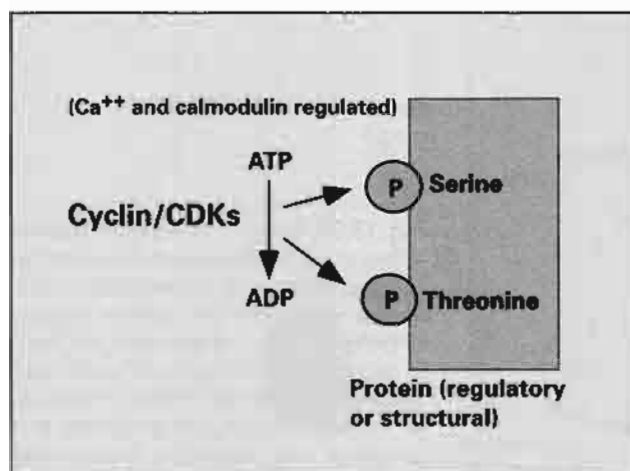


Fig 13-25 Function of cyclin-dependent kinases (CDKs), cytoplasmic enzymes that activate a variety of other enzymatic regulatory proteins by phosphorylation (P) of serine and threonine. The timing, substrate specificity, and site of CDK activity during the cell cycle are determined by cyclins. (ADP) Adenosine diphosphate; (ATP) adenosine triphosphate.

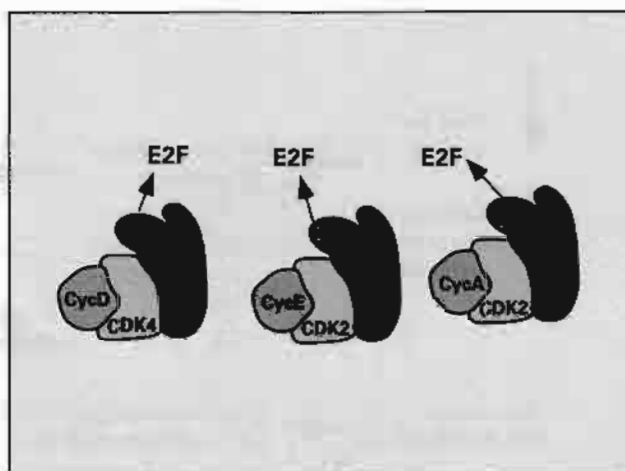


Fig 13-26 Transcriptional regulation at the transition from cell cycle phases G₁ to S. "Pocket proteins" pRb, p107, and p130 bind and inactivate transcription factor E2F until they are heavily phosphorylated by cyclin-dependent kinase (CDK) and cyclins (Cyc). E2F activates genes required for DNA replication. The level of free E2F proteins rises near the end of the G₁ phase and during early S phase.

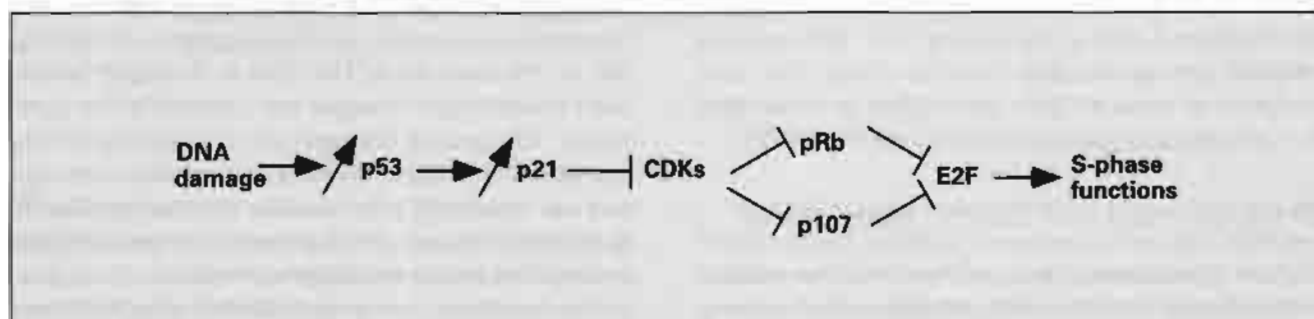


Fig 13-27 Effect of DNA damage on the cell cycle. Damage to DNA blocks the cell cycle by increasing the expression of cyclin-dependent kinase (CDK) inhibitors. Subsequent failure of the phosphorylation of pRb and p107 prevents the release of E2F and the start of DNA replication. If DNA damage becomes uncoupled to the expression of p53 and p21, the chance of developing a malignant cell line increases sharply.

Altering the expression of CDKIs, as in the following examples, can regulate cell division. The tumor suppressor protein, p53, acts to control cell division by increasing the expression of p21.²¹⁵ This forms the basis for the suppression of cell division that results from DNA damage incurred during senescence and after ultraviolet irradiation (Fig 13-27). The stimulation of p53 expression upregulates the production of p21, acting as a blocker of CDK phosphorylation of pocket proteins (pRb and p107) and of the release of E2F, preventing entry into the S phase. Furthermore, p53 plays a key role in activating apoptosis of damaged cells.²¹⁵ Abnormalities in p53 lead to accumu-

lation of genetic damage and may lead to unregulated growth. Mutations in p53 are a common finding in diverse forms of premalignant lesions and cancer.^{216,217}

The regulatory role of TGF- β in controlling cell proliferation is the result of its ability to increase the expression of p27 and p15. Under the influence of TGF- β , epithelial cells remain in G₁ arrest. The mitogenic effect of IL-2 on lymphocytes is in part a result of the ability of IL-2 to inactivate p27, thereby promoting cell division.

Intracellular calcium is an important cytoplasmic component of several signaling pathways that regu-

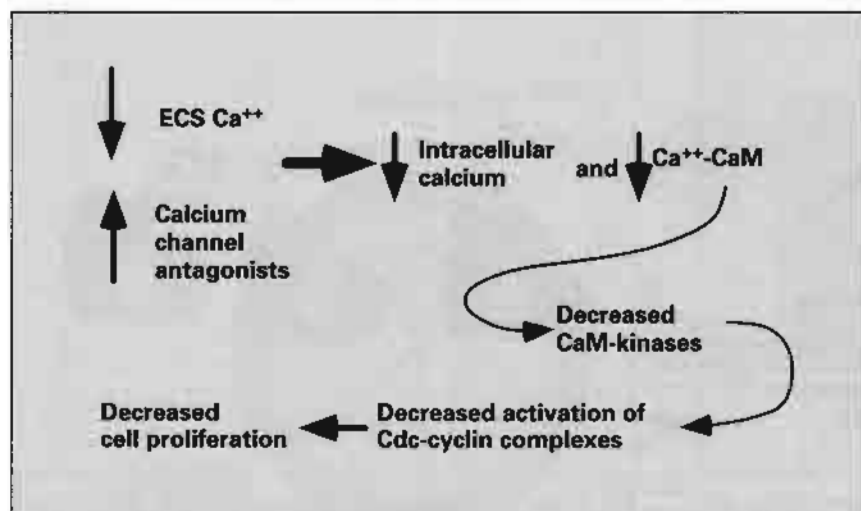


Fig 13-28 Effect of intracellular calcium levels on cell proliferation. A decline in the cytoplasmic calcium concentration either from a reduction of extracellular calcium or the blockage of calcium channels leads to decreased activation of calmodulin and calmodulin-dependent kinases. The phosphorylation of Cdc-cyclin complexes and several of their regulators requires calmodulin-dependent kinase activity. (CaM) Calmodulin; (ECS) extracellular space.

late cell division (Fig 13-28). Interference in the pathway from plasma membrane G protein-coupled receptors, to activation of phospholipase C and the release of Ca^{++} from intracellular stores, decreases cell proliferation. Limiting the entry of Ca^{++} with calcium channel antagonists has a similar effect. The transcription of several CDKs and cyclins is dependent on calmodulin-regulated proteins (see Fig 13-28).

Programmed cell death (apoptosis)

Human development and survival requires mechanisms for precise control of cell populations. During organogenesis, tissue modeling necessitates the pinpoint elimination of specific cells or groups of cells, just as much as it must promote the survival and orderly proliferation of other cell cohorts. In the adult organism as well as in the embryo, it is imperative that cells that have acquired the ability to undergo unregulated growth be killed before they compromise the health and survival of the host. This is accomplished by an all-or-none program of degradative reactions aimed at the cell's genetic material and cytoplasmic components.^{218,219}

Programmed cell death, *apoptosis*, is a rapid process involving individual cells. In contrast, pathologic cell death, or *necrosis*, is usually slower and most often involves groups of cells. The importance of apoptosis in normal embryonic development, maintenance of adult tissues, and prevention of cancer became recognized during the last decade as techniques for the study of gene regulation and signal transduction became available.

The hallmark of the apoptotic process is rapid (1- to 2-hour) fragmentation and condensation of nuclear DNA, coupled with the breakup of the nucleolus.²¹⁸ Chromatin is cleaved by endonuclease between nucleosomes to give rise to DNA fragments containing 180 to 200 base pairs. The DNA is damaged before overt morphologic changes are detected in the cytoplasm. The nuclear changes are followed by shrinkage of the cytoplasm, filament aggregation, and rupture of the cell into smaller dense bodies.²²⁰ Cytoplasmic transglutaminase enzymes are activated to cross-link many cytoplasmic proteins.

The apoptotic cell is immediately phagocytosed by macrophages or other neighboring parenchymal cells.²²⁰ The plasma membrane changes that allow recognition by macrophages include the early translocation of phosphatidylserine from the inner to the outer leaflet of the plasma membrane.²¹⁹ This transfer is carried out by the enzyme phosphatidylserine transferase.

In necrosis, or pathologic cell death, cells usually swell as a result of mitochondrial damage and the loss of high-energy metabolites to maintain ion pumps at the cell membrane. The end point is cell membrane rupture and cell death. During necrosis, proinflammatory mediators are generated, leading to the chemoattraction of inflammatory cells to the site of necrosis.

In contrast, apoptosis has no inflammatory component. One reason that apoptotic cells do not contribute to inflammation is the activation of tissue transglutaminase, a cytoplasmic enzyme that cross-links cytoplasmic proteins.²²⁰ The introduction of ex-

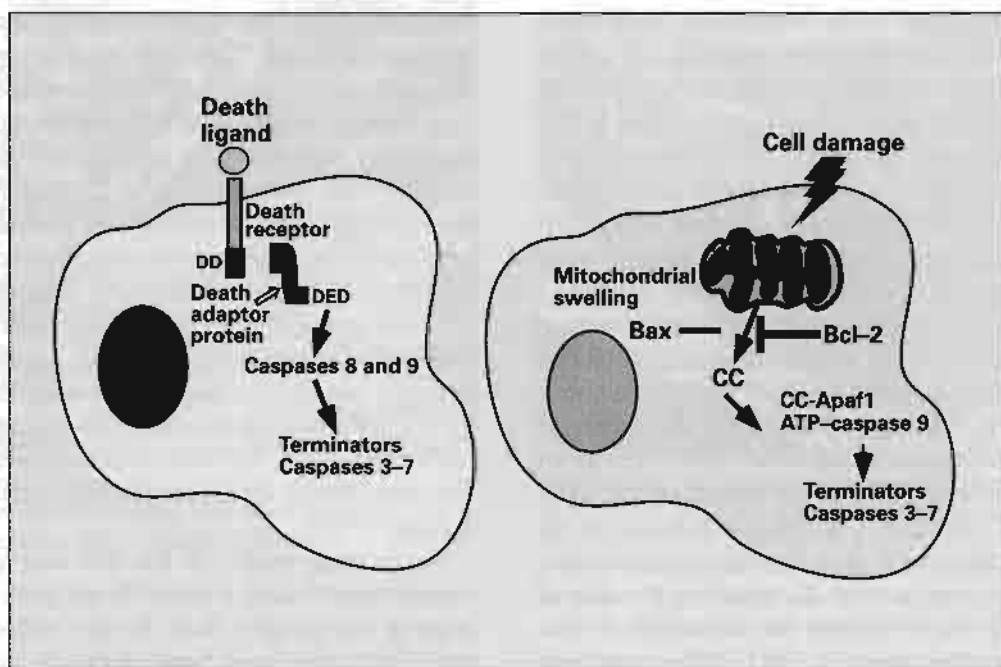


Fig 13-29 Dual pathways for triggering apoptosis. Death ligands, such as Fas ligand, initiate cell death by binding to specific death receptors. Nonspecific cellular stress, leading to mitochondrial damage, causes the release of cytochrome C (CC) from mitochondria, leading to activation of caspase 9. In both pathways, the activation of caspases 8 and 9 causes the activation of the final terminators, caspases 3 to 7. (Apaf1) Apoptosis activating factor 1; (ATP) adenosine triphosphate; (DD) death domain; (DED) death effector domain.

tensive cross-links keeps the cytoplasmic content within the cell and contributes to the shrinkage of cellular components. The activity of tissue transglutaminase is highest in cell fragments (apoptotic bodies).

The signal transduction and gene transcription events involved in apoptosis are far from being completely identified. The process involves stimulatory and inhibitory proximal transduction pathways triggered by cell surface signaling events²¹⁸ (Fig 13-29). The initial or proximal pathways may require new gene transcription. In some cells, stimulatory proximal pathways appear already activated but progression to apoptosis is prevented by the presence of an inhibitor substance, such as Bcl2. In other cell lines, survival depends on the continued presence of growth factors or hormones. Removal of the signaling stimulus generated by the growth factor induces apoptosis. Many of the proteins that regulate cell division (pRb, E2F, and p53) also regulate activation of the programmed cell death pathway.^{209,221} A toxin produced by the periodontal pathogenic bacterium *A. actinomycetemcomitans* induces cell cycle arrest and apoptosis of B-cell lines in culture, suggesting a potential role in progression of periodontal disease.²²²

Several plasma membrane receptors that trigger the apoptotic pathway have been identified. The two receptors that have been most extensively studied are the Fas receptor protein and TNF receptor. The corresponding ligands, Fas ligand and TNF, are known as *death factors*. Several other proteins (all members of the TNF family) have the potential for activating the programmed cell death pathway. The Fas-Fas ligand interaction is believed to be involved in the removal of activated mature T cells once they have completed their immune function. This system is also one pathway for the cytotoxic destruction of target cells by cytotoxic T cells and natural killer cells.

The final steps in the signaling pathway that lead to apoptosis are the activation of several proteases, including caspases and endonucleases (see Fig 13-29). Caspases are cysteine proteases that cut their substrate proteins at sequences containing aspartate.^{219,220} One of the earliest caspase enzymes involved in apoptosis was the interleukin 1 β -converting enzyme.²¹⁸ These enzymes are expressed constitutively as proenzymes. Other proteases, such as granzyme A and calpain, also participate in carrying out apoptosis.²¹⁸

The mitochondrial outer membrane has been shown to act as a control point for caspase activation. Cytochrome C, released from damaged outer mitochondrial membranes, appears to have a key role in the activation of caspases.²¹⁹ Mitochondrial damage induced by various changes in cell metabolism, such as too high a level of calcium, or low pH, precedes the release of cytochrome C through mitochondrial membrane megapores.

Prominent among the many cytoplasmic regulators of apoptosis is Bcl-2, the product of *Bcl2* (B-cell lymphoma/leukemia gene 2).²¹⁸⁻²²⁰ This protein was one of the first members of the large Bcl family of apoptosis regulators to be identified. Bcl-2 acts as an anti-cell-death factor. It is concentrated on the outer mitochondrial membrane, where it acts to block the escape of cytochrome C from the mitochondrial inner space through megapores. By blocking the exit of cytochrome C, Bcl-2 reduces the activation of caspases and apoptosis (see Fig 13-21). Other members of the Bcl family, such as Bax, act to facilitate the exit of cytochrome C from mitochondria and thus increase apoptosis.

The Fas-Fas ligand pathway for inducing apoptosis does not involve mitochondrial alteration and the release of cytochrome C. Activation of Fas stimulates a direct activation of the caspases.

Clinical Correlation: Immune Response in Gingival and Periodontal Disease

The colonization of the tooth surface by a wide variety of microbes, particularly at the cervical part of the crown, in juxtaposition to the permeable junctional epithelium, creates a condition for high levels of antigen stimulation of host cells. Penetration of the junctional epithelium by bacterial mitogens, antigenic polypeptides, and bacterial proteases leads to inflammation of the underlying lamina propria and local activation of innate and acquired immune responses.

There have been many studies of the local population of immune cells in diseased gingival tissues. A goal of these studies has been to determine the respective roles of T and B cells in the pathogenesis of periodontal disease. There is a considerable diversity of opinion as to the potential protective and destructive roles of lymphocytes in the pathogenesis of periodontal disease.²²³ In a recent review of the controversies that exist regarding the role of the im-

munologic response in periodontitis, Gemmell et al²²³ have emphasized the destructive potential of lymphocytes and their inflammatory cytokines in tissue.

It is broadly accepted that the immune response to plaque organisms is predominantly a T-cell response, with both CD4⁺ and CD8⁺ cells infiltrating the local connective tissue and to a lesser degree the intraepithelial spaces of the junctional epithelium²²⁴⁻²²⁸ (Figs 13-30 and 13-31). A large percentage of these are memory and effector cells (CD45RO⁺). However, if the bacterial plaque is allowed to remain in place, over time numerous B cells infiltrate the gingival connective tissue, and plasma cells differentiate locally. Thus, the established gingival lesion and the periodontitis lesion are predominantly T_H2-B-cell lesions.^{25,76,226}

Antibodies, mainly of the IgG and IgA class, directed toward plaque bacterial antigens are found in plasma and gingival fluid. Serum antibody levels to periodopathogens have been shown to increase in patients with periodontitis.²²⁹ Serum IgG antibodies are produced against specific microorganisms contained in plaque. Antibodies to *Porphyromonas gingivalis* and *A. actinomycetemcomitans* are present in the serum and gingival fluid of patients with adult periodontal disease. These antibodies are directed to surface components of the bacterial cells.

In general, antibodies help to protect the host by increasing the phagocytosis of bacteria by macrophages and neutrophils, by interacting with components of the complement system to induce microbial lysis, and by blocking specific virulence factors, such as bacterial proteases. These specific immune responses may limit the total number of bacteria but are incapable of total elimination of microorganisms from the bacterial plaque once large colonies have developed.

There is also evidence that in the early stages of gingivitis and periodontal disease the bulk of the T helper cells are of the T_H1 class. These cells secrete IFN- γ and IL-2 and stimulate macrophages to secrete IL-1. Macrophages are also a prominent cell type in the inflamed tissues.²³⁰ In chronic lesions, the T-cell population shifts toward the T_H2 class, with increased expression of IL-4, IL-5, and IL-6.^{25,77,231} These cytokines are important in supporting B-cell differentiation. Some investigators suggest that T_H1 dominance leads to a more aggressive lesion with significant bone resorption and decreased bone formation because of the higher levels of IL-1 and IL-2 production.²³² On the other hand, the T_H2 phase, with its elevated secretion of B-cell stimulatory interleukins, is thought to be protective.

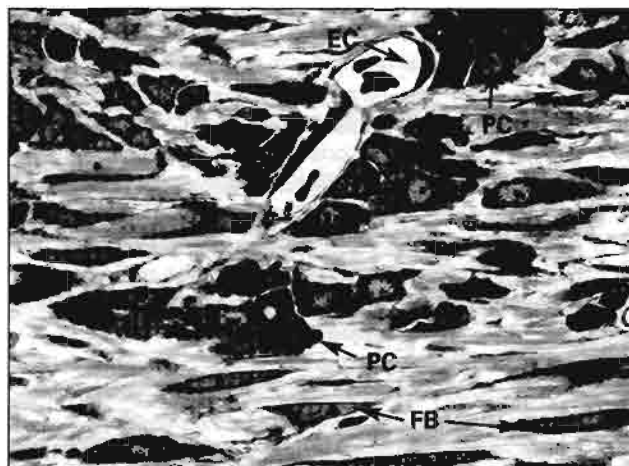


Fig 13-30a Inflammatory cell infiltration of gingival connective tissue in periodontal disease. (EC) Endothelial cell; (FB) fibroblast; (PC) plasma cell. (Original magnification $\times 1,600$.)

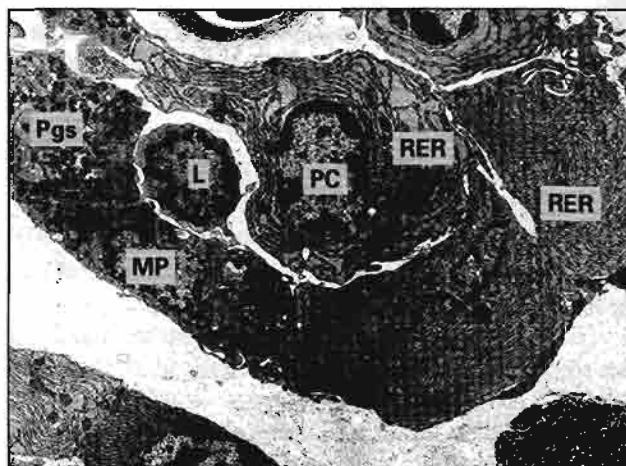
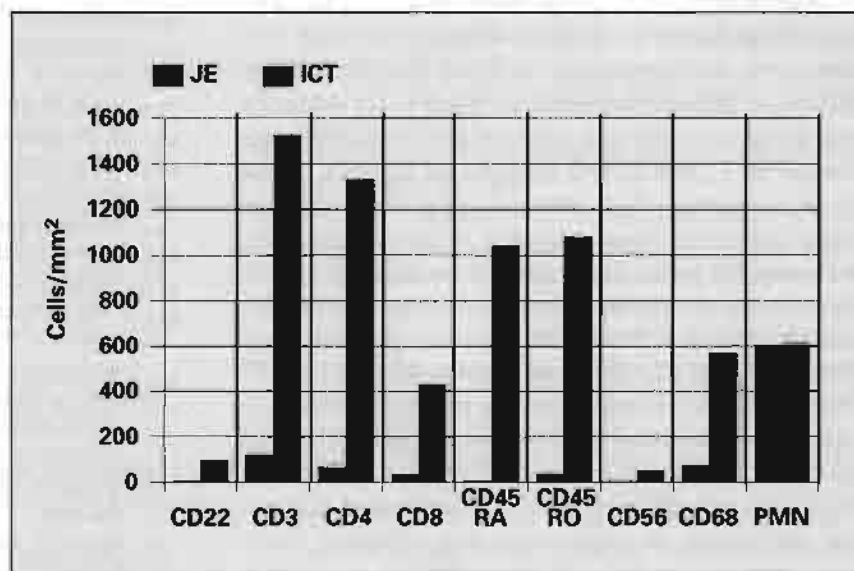


Fig 13-30b Cell clusters in the inflamed gingiva. The cell clusters contain plasma cells (PC), characterized by their chromatin pattern and abundance of rough endoplasmic reticulum (RER); macrophages (MP) containing numerous phagosomes (Pgs); and lymphocytes (L), characterized by their small cytoplasmic volume. (Original magnification $\times 3,600$.)

Fig 13-31 Leukocyte composition of the gingival infiltrate, expressed in cells per square millimeter of junctional epithelium (JE) and infiltrated connective tissue (ICT) of clinically normal gingiva. Cell identification is by surface markers: (CD22) B cells; (CD3) T cells; (CD4) helper T cells; (CD8) cytotoxic/suppressor T cells; (CD45RA) naive cells; (CD45RO) memory or activated cells; (CD56) natural killer cells; (CD68) monocytes and macrophages; (PMN) polymorphonuclear neutrophils. This degree of immune response is viewed as "effective coping" to a low level of chronic antigenic stimulation present in the gingival environment. (Adapted with permission from Tonetti.²²⁶)



Porphyromonas gingivalis lipopolysaccharide can induce polyclonal activation of B cells via the stimulation of surface pattern recognition receptors.¹¹⁰ Polyclonal activation of T cells by superantigens in periodontally diseased tissue has been suggested from the genetic analysis of the expression of TCR proteins. Endotoxin-activated T cells exert a stimulatory effect on osteoclastic alveolar bone resorption.²⁵

Autoantibodies to collagen have been detected in periodontitis lesions.^{233,234} Experimental in vitro studies have shown that polyclonal B-cell activators in the

form of lipopolysaccharide, when combined with type I collagen molecules, lead to formation of anticollagen antibody-forming cells.²³⁵ The presence of anticollagen antibodies and free collagen fragments in chronically inflamed gingival tissues could contribute to the pathogenesis of periodontitis by formation of immune complexes and the activation of complement.

Gingival inflammatory infiltrates contain CD4⁺ and CD8⁺ memory primed T cells expressing the $\alpha\beta$ and $\gamma\delta$ TCRs.^{236,237} It has been determined that some of

these lymphocytes are activated to specific periopathogen antigens.²³⁸ Analysis of the expression of the variable β chain regions in gingival lymphocytes showed some skewing suggestive of possible superantigen activation. In rapidly progressive periodontitis lesions, the pocket connective tissue contains CD4⁺CD8⁺ cells in a ratio of 1:12 and large aggregations of plasma cells.²³⁹ Natural killer cells are also present, increasing in numbers in more severe lesions.^{240,241}

Cytolytic T cells have been found to make up about 10% to 20% of all lymphocytes in the gingival infiltrate. Most of these cells have $\gamma\delta$ TCRs and are concentrated in the lower layers of the pocket epithelium.²⁶ The possibility that these cells exert a cytotoxic attack on gingival epithelial cells and fibroblasts has been proposed, but the evidence remains inconclusive. It is perhaps more likely that they perform a regulatory role for epithelial cells and for limiting T- and B-cell activity.²⁴ It has been proposed that $\gamma\delta$ T cells may promote epithelial cell survival by producing keratinocyte growth factor.¹³⁷

Macrophages and Langerhans cells carry out antigen presentation in gingival tissues. Although keratinocytes and fibroblasts increase their expression of MHC-II during inflammation, there is no evidence that either cell type can support antigen-specific activation of T cells during periodontal disease. It has been suggested that, although gingival fibroblasts have increased levels of class II MHC molecules, they may fail to function as APCs because of insufficient levels of costimulation.²⁴² On the other hand, recent studies indicate that human gingival fibroblasts are able to present superantigens to T cells.²⁴² This has led to the idea that they may play a role in T-cell activation during colonization by certain types of bacteria. Fibroblasts and keratinocytes support local infiltration and differentiation of lymphocytes through the expression of interleukins and adhesion molecules and the secretion of matrix ligands, such as fibronectin, for lymphocyte migration.^{243,244}

Among the hundreds of different species of oral microorganisms, about six species have been associated with periodontal disease. *Porphyromonas gingivalis* appears to be the most pathogenic. Because of the relatively small number of pathogenic species, it has been proposed that a vaccine might prevent periodontal diseases. Protective antibodies might prevent or limit the colonization of tooth surfaces before large colonies can form. Results obtained in experimental animals have shown that immunization to *P. gingivalis* fimbrial protein does lead to a reduction in bone resorption and connective tissue proteolysis in animals subsequently infected with *P. gingivalis*.

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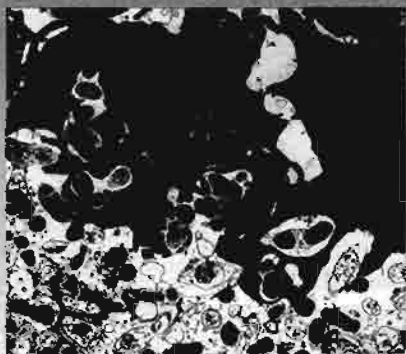
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Chapter 14

Phagocytic Cells



The mucosal surfaces of the oral cavity, as well as those of the entire gastrointestinal tract, are continuously exposed to very high numbers of bacteria. Fortunately the great majority of these microorganisms live in a symbiotic relationship with the host. The relatively low incidence of bacterially induced pathosis within the oral epithelium and in its underlying connective tissue is the result of many factors, including the relative impermeability of stratified squamous epithelium to water-soluble substances and its high rate of renewal.

A further line of defense lies in a rapidly deployable system of phagocytic cells whose chief functions are the ingestion and destruction of invading organisms. The polymorphonuclear neutrophil (PMN) is the key responder in this system. It is a suicide cell programmed to detect and home to foreign organisms (chemotaxis), capture them (adhesion), ingest them (phagocytosis), and destroy them by exposing them to reactive oxygen metabolites (ROMs) and to a battery of lytic enzymes. Activated PMNs also express regulatory cytokines that can modulate various aspects of the inflammatory process.

The monocyte-macrophage cell line also participates in this defense system. These cells not only phagocytose and destroy foreign organisms and their products but also play major roles in the efferent and afferent arms of the immune system. Macrophages manufacture proteins of the complement system. They also secrete a variety of regulatory cytokines and

proteolytic enzymes that are important not only during an inflammatory response but also during normal connective tissue remodeling.

Because of the short life span of phagocytic cells, there must be a constant production of replacement cells from bone marrow stem cells. Various growth factors and cytokines regulate their proliferation and exit from bone marrow to blood. The level of circulating neutrophils and mononuclear phagocytes increases above normal limits (a condition known as *leukocytosis*) in response to various inflammatory diseases.¹ This chapter will focus on the mechanisms of exit from the bloodstream and the biologic action of phagocytic cells at the site of disease or injury.

Under normal conditions, phagocytic cells function subclinically, responding to a background level of bacterial intrusion. However, when phagocytic cells are activated in large numbers, their potent destructive nature can act as a double-edged sword, destroying host cells and tissues during an inflammatory response.^{2,3} The proinflammatory effect of neutrophil activation is produced by the production and release of cytokines, prostaglandins, leukotrienes, matrix metalloproteinases (MMPs), cathepsins, reactive oxygen metabolites, and nitric oxide (Fig 14-1).

The importance of the constant presence of phagocytes is revealed by the life-threatening diseases that develop in patients who have genetic defects in these cells.⁴

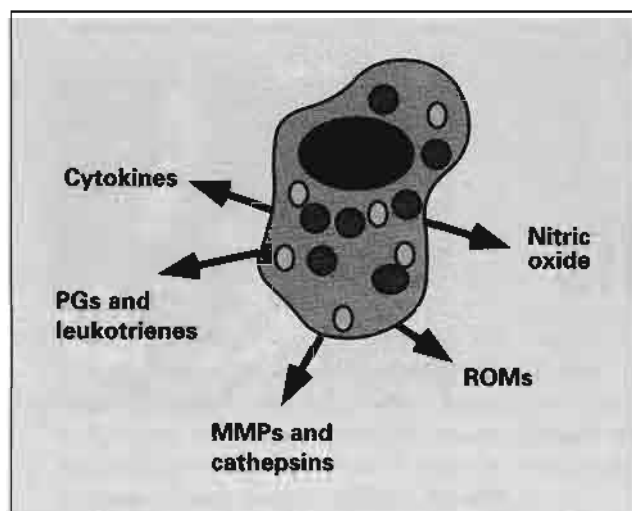


Fig 14-1 Activated neutrophil. The proinflammatory effect of neutrophil activation is produced by the production and release of cytokines, prostaglandins (PGs), leukotrienes, matrix metalloproteinases (MMPs), cathepsins, reactive oxygen metabolites (ROMs), and nitric oxide.

Development of Polymorphonuclear Neutrophils

Polymorphonuclear neutrophils develop from bone marrow stem cells that have been stimulated by granulocyte-monocyte colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor. Stromal cells of the bone marrow contribute interleukin 1 (IL-1) and interleukin 3 (IL-3), promoters of the differentiation process.

In their developmental stage, PMNs contain rough endoplasmic reticulum and well-developed Golgi complexes. The hallmark of the mature PMN is its complement of specialized lysosomal granules.⁵ At least three types of storage granules are formed from the Golgi apparatus. The first to form are large azurophilic granules, 0.5 to 0.8 μm in diameter. They contain elastase, acid hydrolases, lactoferrin, lysozyme, β -glucuronidase, defensins, and myeloperoxidase. Smaller, specific or secondary granules are the next to develop. They contain lactoferrin, cytochrome *b*, collagenase (MMP-8), gelatinase (MMP-9), and the Mac-1 (CD11b-CD18) integrin. Tertiary granules, the last to form, are narrow, elongated structures that contain gelatinase, H^+ -adenosine triphosphatase, cytochrome *b*, and Mac-1.⁶ Small secretory vesicles also contain integrins, alkaline phosphatase and components of the oxidase system.

A spectrum of receptor proteins, such as the receptors for *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), fibronectin, laminin, and plasminogen activator, are contained in the secondary and tertiary gran-

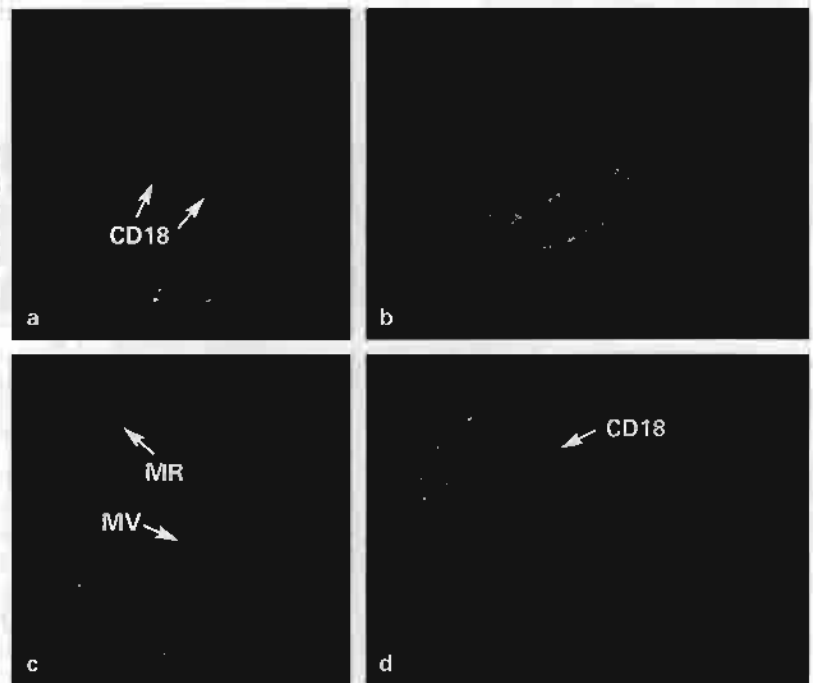
ules and the secretory vesicles.⁵ In general, the specific and tertiary granules contain receptors for recruitment to sites of inflammation, components of the oxidase system, and extracellular matrix-degrading enzymes. In contrast, the azurophilic granules contain mainly microbial killing enzymes and agents.^{5,7}

As the full complement of granules nears completion, the rough endoplasmic reticulum and Golgi compartments undergo rapid decline. During maturation, energy is stored in glycogen particles (30 to 40 nm in diameter). Because mature PMNs have very few mitochondria, energy, in the form of adenosine triphosphate, must be generated by the breakdown of glycogen particles (glycolysis). This enables the PMNs to function in poorly oxygenated areas of tissue damage.

Cell division occurs at least four times during PMN development. With each mitotic division, the shape of the nucleus is modified until it ultimately assumes a highly lobulated shape. In the mature PMN, the nucleus has three to four lobules joined by short, thin strands of nuclear matrix. Only eosinophils and basophils achieve a similar, albeit lesser, nuclear lobulation. It has been suggested that the highly lobulated nucleus of the PMN facilitates cell migration through narrow intercellular spaces and small breaches in the basal lamina.

Newly developed PMNs enter venous sinuses of the bone marrow to join a circulating pool of 60 trillion PMNs. Time spent in the bloodstream is only about 6 to 7 hours. Polymorphonuclear neutrophils exit the circulation following stimulatory adhesive interactions with specific proteins displayed on endothelial surfaces. A large percentage of PMNs exit

Figs 14-2a to 14-2d Scanning electron micrographs of polymorphonuclear neutrophils. (a, b) Unstimulated cells. (Original magnification $\times 1000$ and $\times 500$, respectively.) (c, d) Cells stimulated with *N*-formyl-methionyl-leucyl-phenylalanine (fMLP). (MR) Microridge; (MV) microvilli. (Original magnification $\times 1000$ and $\times 500$, respectively.) In (b) and (d), a $\beta 2$ integrin, CD18, is localized with a monoclonal antibody conjugated to colloidal gold particles. In (d), note the change in cell shape and the polarized distribution of CD18 in response to the chemoattractant properties of fMLP. (Adapted from Fernandez-Segura et al⁹ with permission from Elsevier Science.)



the circulation through submucosal blood vessels, especially those located in the lamina propria of the intestines.

After exiting the bloodstream, PMNs live for no more than 2 to 3 days. During this time, the cell migrates through tissue spaces in response to various chemotactic gradients. During migration, the cell has a polarized shape. The nucleus is located in the trailing end of the cell, while the centrosome and elements of a reduced Golgi apparatus are positioned between the nucleus and the leading edge of the cell. The leading cytoplasmic process (uropod) is characterized by a rich network of F-actin filaments and polarization of integrins (CD18) toward the frontal pole (Figs 14-2a to 14-2d).^{8,9} Uropod formation occurs within seconds following the exposure of PMNs to strong chemoattractants such as IL-8 and platelet-activating factor.¹⁰

Following several days of activity, the PMN enters a programmed cell death pathway and ultimately undergoes phagocytosis and degradation by macrophages. The ligation of CD11a and CD11b integrins during endothelial transmigration delays spontaneous PMN apoptosis.¹¹ Both GM-CSF and tumor necrosis factor (TNF), produced locally by helper T (T_H 1) cells, can prolong the survival of PMNs by decreasing the rate of apoptosis.¹² In contrast, IL-10 exerts an anti-inflammatory effect by promoting apoptosis of PMNs and by

decreasing the recruitment of new PMNs by down-regulating the expression of IL-8.¹³ Apoptosis of PMNs occurs following integrin-mediated phagocytosis in a mechanism involving the formation of reactive oxygen metabolites.¹⁴

Role of Polymorphonuclear Neutrophil Cell Surface Receptors

The plasma membrane of the PMN contains myriad receptors that monitor the surrounding milieu for the presence of molecules that signal a call to action (Fig 14-3). Following appropriate receptor-ligand interaction, PMNs undergo partial activation (priming), leading to polarization and chemotaxis, or full activation for phagocytosis and degranulation. Receptors for IL-8, C5a, and fMLP are potent activators of chemotaxis.¹⁵ Complement receptors and Fc receptors play important roles in phagocytosis.¹⁶ The $\beta 2$ integrins (leukocyte function antigen 1 [LFA-1] and Mac-1) and the hyaluronate receptor (CD44) interact with matrix ligands during transit through endothelium and chemotaxis,^{17,18} while CD14 is a receptor for lipopolysaccharide (LPS) and other surface components of bacteria.¹⁹

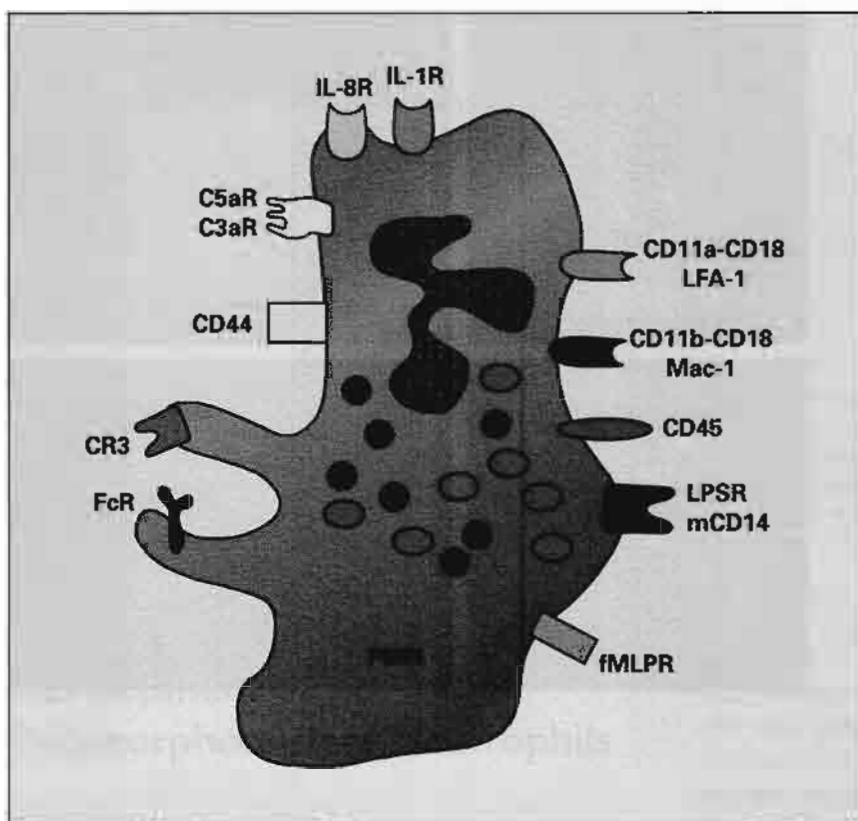


Fig 14-3 Plasma membrane receptors expressed by a polymorphonuclear neutrophil (PMN). The receptor for the Fc component of antibodies (FcR) and the complement receptor (CR3) permit attachment of antibody and C3b opsonized particles. Receptors for C3a (C3aR) and C5a (C5aR) function in chemotaxis and activation of the PMN. Leukocyte function antigen 1 (LFA-1) and Mac-1, both members of the CD18 family of integrins, and the hyaluronate receptor (CD44) serve as matrix attachment sites during transmigration from blood vessels as well as during chemotaxis ($\beta 1$ integrins not shown). Receptors for lipopolysaccharide (LPSR) and *N*-formyl-methionyl-leucyl-phenylalanine (fMLPR) initiate activation of PMNs. Additional stimulation and activation of gene expression is triggered by receptors for interleukins 1 (IL-1R) and 8 (IL-8R), the latter especially active in chemotaxis of PMNs. (mCD14) Cell membrane CD14.

Surface receptors are intimately associated with cytoskeletal elements in the cortical cytoplasm through complex signal transduction pathways. Redistribution of receptors occurs during cytoplasmic polarization in chemotaxis and phagocytosis.⁸ For example, following PMN chemotactic activation, CD18 ($\beta 2$) integrins concentrate in cell processes at the leading edge of the cell, presumably creating tractional advantage at that location (see Figs 14-2a to 14-2d).

Activation of Polymorphonuclear Neutrophils

As the PMN enters the functional stages of its brief life span, it may undergo several degrees of stimulation, from priming reactions leading to transmigration from the bloodstream to full activation during phagocytosis and degranulation.^{20,21} Each of these phases is initiated and regulated by receptor-ligand interactions and their associated signaling cascades. Endogenous stimulatory ligands, such as chemokines, cytokines, immunoglobulins, and complement, and products of exogenous origin, such as bacterial LPS, bind to cell

surface receptors and activate PMN responses. Lipopolysaccharide, TNF- α , platelet-activating factor, and complement components are among the most frequently encountered priming agents. High concentrations of LPS and TNF- α can trigger granule secretion and subsequent tissue damage.

When present at low concentrations, many activating substances stimulate PMN transmigration but do not trigger granule secretion or a respiratory burst. During activation, the expression of integrins is increased and their binding affinity is heightened.^{22,23} Integrin binding to their extracellular matrix ligands has been shown to exert a regulatory effect on cytokine receptor expression by PMNs.^{24,25} In general, integrin binding has a stimulatory effect on PMNs and monocytes. Actin filaments increase in number, and the neutrophils develop polarity by concentrating integrins at the leading surface.²²

More potent activators of PMNs include chemotactic factors such as bacterial fMLP, IL-8, leukotriene B₄, platelet-activating factor, and the complement component C5a. In addition to acting as chemoattractants, these substances also trigger respiratory bursts and degranulation.²⁶ The IL-8 receptor is a

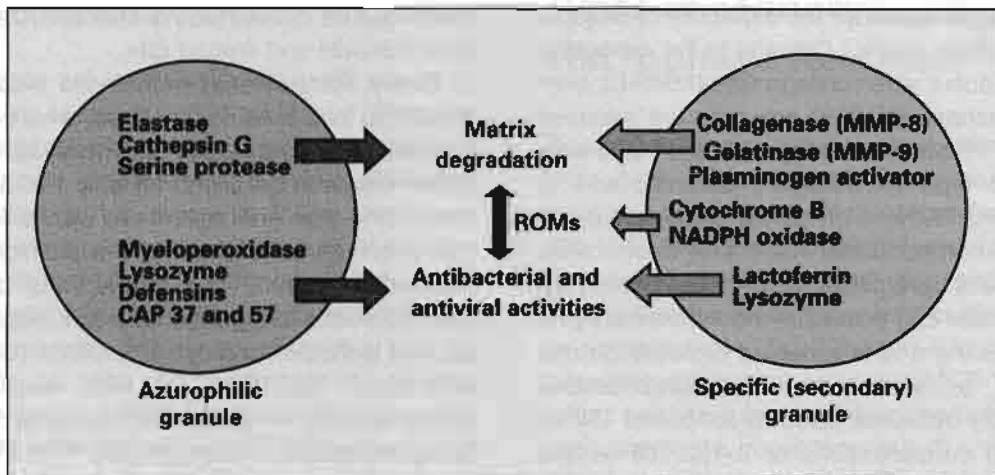


Fig 14-4 Partial content of the primary and secondary granules of the polymorphonuclear neutrophil. Release of granular contents during activation and phagocytosis provides protection against bacterial and viral infection but can also lead to significant connective tissue degradation. The generation of reactive oxygen metabolites (ROMs) can potentiate the helpful as well as the harmful effects of degranulation. (CAP) Cationic protein; (MMP) matrix metalloproteinase; (NADPH) nicotinamide adenine dinucleotide phosphate.

seven-pass transmembrane protein coupled to heterotrimeric guanosine triphosphate (GTP)-binding proteins.²⁷ When IL-8 attaches to its receptor, it causes GTP to bind to the α_s stimulatory component of the heterotrimeric GTP-binding protein, and subsequent activation of the Ras-GTP and mitogen-activated kinase signaling pathways. The $\gamma\delta$ components of the heterotrimeric GTP-binding protein activate phospholipase C β , leading to a rise in the cytosolic Ca^{++} concentration. The expression of β_2 integrins is upregulated following IL-8 activation. Other mediators of PMN activation that act via GTP-protein-linked receptors include GM-CSF, LPS, TNF- α , and C5a and C3a receptors.¹⁶

Bacterial surface components are powerful activators of neutrophils, monocytes, and macrophages. Gram-negative bacteria shed surface glycolipids or lipopolysaccharides that activate PMNs and macrophages. Lipopolysaccharides bind to cell membrane CD14 (mCD14), a peripheral membrane protein found on several cell types, including PMNs, macrophages, and fibroblasts. Soluble CD14 (sCD14) in serum and tissue fluids binds LPS to LPS-binding protein (LBP). These LPS-LBP-CD14 complexes interact with other Toll-like receptors on the cell surface, activating signal pathways involving tyrosine phosphorylations and the subsequent stimulation of mitogen-activated protein kinases. In vitro studies show that, in the case of LPS-induced activation of PMNs, internalization of LPS-CD14 complexes within endosomes precedes signal

transduction events. The structure of LPS and its interaction with various cell types is discussed in chapter 13. Activation prepares the neutrophils for phagocytosis and microbial killing.

Degranulation, the loss of cytoplasmic granules, results from translocation and exocytosis of granules into phagosomes or into the extracellular milieu during unsuccessful attempts to engulf large objects ("frustrated" phagocytosis).²⁸ Granule discharge is preceded by cytoskeletal rearrangement involving signal transduction pathways that regulate actin filament, intermediate filament, and microtubule assembly and disassembly.²⁹

The earliest PMNs to arrive at a site of bacterial or viral infection, or tissue injury, recruit additional PMNs and monocytes by the release of the IL-8 chemokine. Recent evidence indicates that IL-8 messenger RNA is present in nonactivated PMNs and that IL-8 is the only cytokine that can be quickly released on activation.³⁰ Other cytokines, such as IL-1, IL-6, and TNF- α , require gene transcription and a time lag of about 24 hours before they can be secreted. However, only small quantities of these cytokines are produced by PMNs because of their short life span. The early secretion of IL-8 by PMNs could lead to paracrine activation of monocytes and endothelial cells.

Polymorphonuclear neutrophil granules contain a wide variety of biologically active substances that can destroy bacteria and viruses³¹ (Fig 14-4). Neutrophils

and macrophages also have the capability of destroying the extracellular matrix.² Damage to the extracellular matrix can occur when collagenase (MMP-8), elastase, and gelatinase (MMP-9) enzymes are released and activated.^{32,33} Matrix metalloproteinase 9 and elastase degrade type IV collagen, laminin, and fibronectin, aiding PMNs in breaching basement membranes.³⁴ Polymorphonuclear neutrophil membrane-bound elastase is upregulated by proinflammatory cytokines.³⁵ Elastase can potentiate the inflammatory response by cleaving a wide variety of extracellular matrix proteins, as well as by activating several proinflammatory cytokines, such as IL-1 β and TNF- α . Local cytokine concentrations of IL-1 β , TNF- α , and GM-CSF differentially regulate the release and activation of MMPs for leukocyte migration and tissue destruction.³⁶ Both the protective (antibacterial) and the tissue-damaging effects of the granule contents are potentiated by the production of reactive oxygen metabolites by activated PMNs.^{31,37}

Evidence that PMN enzymes are involved in periodontal tissue destruction comes from the finding that they are present in the crevicular fluid of periodontitis patients at significantly higher concentrations than in the fluid collected from gingivitis patients and healthy controls.^{33,38} Furthermore, early studies of PMN-bacterial interactions *in vitro* demonstrated that Gram-positive periodontopathic bacteria induced the release of lysosomal enzymes into the culture fluid.³⁹

Intravascular priming (weak activation) of circulating neutrophils has been shown to be a risk factor for cardiovascular disease.⁴⁰ It has been suggested that local infections (including periodontal disease) and smoking cause slight elevations of activating factors in plasma, leading to PMN priming and atherosclerotic changes in blood vessels.^{41,42} Priming factors generated in periodontal disease include LPS, IL-1 β , TNF- α , and prostaglandin E₂.^{43,44}

Development and Structure of Monocytes and Macrophages

The primary site of origin for the monocyte-macrophage cell line is the bone marrow. Pluripotent stem cells are stimulated to develop into monocytes by IL-1, IL-3, and GM-CSF and macrophage colony-stimulating factor (also known as CSF-1).^{45,46} Monocytes are round cells approximately 10 to 12 μ m in diameter. They possess a well-developed Golgi apparatus, numerous lysosomal granules and mitochondria, and an eccentrically placed kidney-shaped

nucleus. The cell surface is characterized by numerous microvilli and coated pits.

Newly differentiated monocytes enter the bloodstream to populate distant sites, where they mature into tissue macrophages and dendritic cells. Monocytes remain in the blood for only 1 to 3 days before interacting with endothelial cell adhesion molecules that promote attachment and transmigration to the connective tissue. The interactions of monocyte CD15 (Lewis X antigen), L selectin, platelet-endothelial cell adhesion molecule 1, LFA-1 (CD11a-CD18), and Mac-1 (CD11b-CD18) with receptors on endothelial surfaces appear to be key factors in regulating monocyte transmigration.^{18,47,48} Following cytokine activation of endothelial cells and marginated monocytes, there is an increase in the surface display and binding avidity of integrins, resulting in monocyte transmigration and concentration at sites of inflammation. After making their exit from venules, monocytes are unable to return to the blood.

Once in the connective tissues, monocytes continue to mature until they acquire the myeloid dendritic cell phenotype or the macrophage phenotype. Endothelial cells have been shown to direct monocyte to myeloid dendritic cell conversion.⁴⁹ The decision to undergo terminal differentiation into a myeloid dendritic cell or a macrophage appears to be regulated by the local cytokine environment. Lipopolysaccharide, GM-CSF, and IL-4 drive immature monocytes toward dendritic cell differentiation. In the absence of these factors, and/or in the presence of macrophage colony-stimulating factor, the macrophage pathway is selected.⁵⁰

During the maturation of macrophages, several surface receptors, such as Fc receptor, vitronectin receptor, and transferrin receptor, are upregulated.⁵¹ Macrophages may live up to several months to perform a wide variety of functions. Some functions involve normal tissue and cell turnover, while other functions are called on as part of the inflammatory and/or immune response.⁵²

Lymphoid dendritic cells constitute a separate line of antigen-processing cells from the myeloid or connective tissue dendritic cell. Lymphoid dendritic cells are derived from a primitive cell that is the ancestor to T and B cells and natural killer cells. Because of the importance of dendritic cells in initiating and regulating immune reactions, they are receiving increasing attention.^{52,53} Myeloid dendritic cells of connective tissue (see also Langerhans cells of epidermis and oral mucosa, discussed in chapter 13) capture soluble and particulate antigen and migrate to regional lymph nodes, where they initiate

immune responses by interacting with antigen-reactive T cells.

Macrophages are larger cells, up to 60 μm in diameter, usually somewhat elongated and often exhibiting polarity. The cell surface contains microvilli and coated pits. Coated vesicles and elongated smooth vesicles are abundant in the cortical cytoplasm. These structures reflect a high level of endocytosis. The hallmark of the macrophage is its large number of lysosomal granules. Older macrophages contain numerous phagosomes and residual bodies, the remnants of past phagocytic activity. Macrophages may undergo fusion to form multinucleated giant cells in a process involving CD44-mediated cell-to-cell adhesion.⁵⁴

Monocytes and macrophages are preferentially located in perivascular sites in the connective tissue. In general, the connective tissue in these sites is typically a loose connective tissue with increased hydration and less densely packed collagen fibrils. Confocal immunofluorescence localization of major histocompatibility complex type II (MHC-II) molecules in dental pulp revealed the presence of dendritic cells around blood vessels.⁵⁵ Studies of dental pulp dendritic cells indicate that they are capable of providing costimulation of T cells.⁵⁶

Monocytes, dendritic cells, and macrophages perform many functions essential to the homeostasis and defense of the host. Dendritic cells play an important role in the activation and regulation of the immune response by acting as antigen-presenting cells and as producers of stimulatory cytokines.⁵⁷ Macrophages perform a protective role by phagocytosing and destroying certain microorganisms. They phagocytose dying cells and damaged components of the extracellular matrix.⁵⁸ Macrophages restrict the growth of cancers by attacking cancer cells. During inflammation and in wound repair, they secrete matrix-degrading enzymes.

To carry out its varied functions, the macrophage produces and secretes nearly 100 regulatory and enzymatic proteins⁵⁹:

1. Colony-stimulating factors
2. Complement factors
3. Coagulation factors
4. Cytokines
5. Growth factors
6. Matrix adhesion molecules
7. Matrix metalloproteinases
8. Prostaglandins
9. Protease inhibitors
10. Reactive oxygen metabolites

Types of Monocyte and Macrophage Receptor

To perform its various functions, the mononuclear phagocyte responds to stimulatory molecules through a wide spectrum of cell surface receptors⁶⁰ (Fig 14-5). Receptors for complement and for the Fc component of antibodies are responsible for the efficient phagocytosis of opsonized bacteria. Receptors for complement C3a, C3b, and C5a and for monocyte chemoattractant protein 1 (MCP-1) activate signaling pathways that regulate chemotaxis of monocytes. Interleukin receptors for IL-1, IL-2, IL-4, and IL-6 activate specific responses. The LPS-binding protein receptor (mCD14) is an important marker protein and activator of cytokine production in monocytes and macrophages.⁶¹

Integrins constitute a substantial component of the receptor population. Adhesion to substrate matrix proteins is mediated by $\beta 1$ integrin (CD29) family members, such as the binding of very late activation (VLA-1 [CD49a-CD29] and VLA-2 [CD49b-CD29]) integrins to collagen. Attachment to fibronectin is mediated via the VLA-4 (CD49d-CD29) and VLA-5 (CD49e-CD29) integrins. Mononuclear phagocytes also have the ability to attach to laminin via VLA-3 (CD49c-CD29) and VLA-6 (CD49f-CD29). The $\beta 1$ class of integrins is needed for monocyte adhesion to extracellular matrix molecules during migration within connective tissues. Integrins of the $\beta 2$ family, such as LFA-1 (CD11a-CD18) and Mac-1 (CD11b-CD18), are important during transmigration through the endothelial wall and in nonopsonic phagocytosis.^{18,62,63}

Fc receptors that bind antigen-antibody complexes and antibody-coated microbes and viruses constitute another pathway of macrophage-neutrophil activation for phagocytosis.⁶⁴ There are many forms of Fc receptors; some lead to activation via immunoreceptor tyrosine-based activation motif (ITAM) units, like those on the CD3 complex of lymphocytes (see chapter 13), while others have an inhibitory action via immunoreceptor tyrosine-based inhibition motif units.⁶⁵ Based on the balance between expression of stimulatory and inhibitory Fc receptors, the binding of antibody-antigen complexes by phagocytic cells can generate inflammatory reactions of varying intensity. Immune complex activation of phagocytic cells leads to the release of proinflammatory cytokines and chemokines.

Macrophages also have scavenger receptors capable of binding a wide variety of ligands, including denatured collagen types I and III, bacterial cell wall

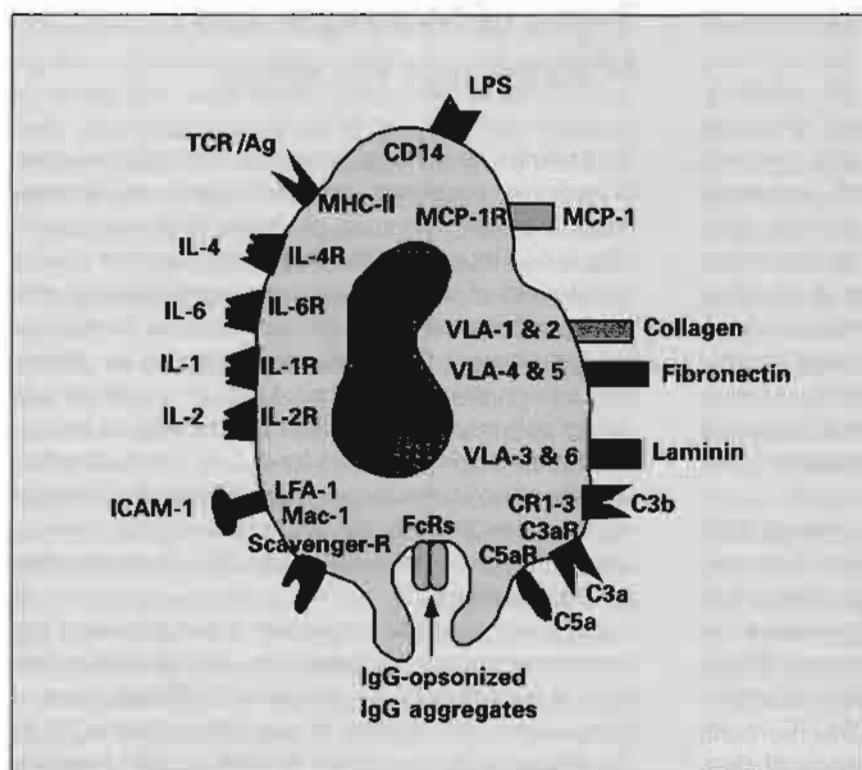


Fig 14-5 Representative macrophage receptors and their respective ligands: (CD14) lipopolysaccharide (LPS) receptor; (MCP-1R) receptor for macrophage chemoattractant protein 1; (CR1-3, C3aR, and C5aR) receptors for complement factors; (FcR) receptor for the Fc component of the antibody; (IL-Rs) various interleukin receptors; (MHC-II) major histocompatibility complex type II, the antigen-presenting molecule that acts as a receptor for T-cell receptor-antigen (TCR/Ag) complex; (Mac-1 and LFA-1 [$\beta 1$]) Mac-1 and leukocyte function antigen 1, integrins that act as receptors for intercellular adhesion molecule 1 (ICAM-1); (VLAs) very late activation molecules, $\beta 2$ integrins that act as receptors for various extracellular matrix proteins, (IgG) Immunoglobulin G.

components, and low-density lipoprotein. Unfortunately, the ability of macrophages to bind low-density lipoprotein leads to the deposition of cholesterol and the formation of atherosclerotic lesions.

The antigen-presenting cell function of macrophages is accomplished by presentation of processed antigen peptides on MHC-II molecules for interaction with antigen-specific T-cell receptor molecules. Macrophages are also capable of processing exogenous antigen in phagosomes and delivering antigenic peptides to MHC-I proteins via the cytoplasmic-proteasome pathway.⁶⁶ The structure of MHC molecules and the topic of antigen processing are discussed in chapter 13.

Additional receptors include scavenger receptors, CD68 (a sialic acid-binding lectin) and mannose receptors.⁶⁰ Scavenger receptors are upregulated by anti-inflammatory cytokines (IL-6 and IL-10).⁶⁷

Activation of Monocytes

Monocytes and macrophages can be stimulated to increased levels of activity (activated) by numerous factors produced by resident cell types, such as keratinocytes, endothelial cells, and fibroblasts, as well as by activated T cells. Bacterial LPS and cell wall

peptides are also potent activating substances.⁶⁸ Interferon γ (IFN- γ) produced by T cells, is a key activating agent of mononuclear phagocytes. Furthermore, GM-CSF and TNF- α have an agonistic effect, potentiated by IL-2 and IL-4, products of $T_H 1$ cells.⁶⁹ Once activated by a specific cytokine, macrophages become unresponsive to additional stimulation by other cytokines.⁷⁰

In vitro studies show that CSF-1 and LPS activate phosphorylation of related adhesion focal tyrosine kinase and downstream signaling via phosphatidylinositol 3 kinase and Ras.⁷¹ This cytokine-activated pathway appears important in regulation of matrix adhesion, migration, and macrophage cell morphology. Of related significance is the observation that fibronectin engagement of fibronectin receptors ($\beta 1$ integrins) increases the phagocytic response of macrophages.⁷²

Activated monocytes and macrophages demonstrate many of the same functions observed in activated PMNs, such as a respiratory burst and the production of ROMs.⁷³ Phagocytic, cytolytic, and bacteriolytic potentials are all increased.⁷³ Furthermore, the potential for local tissue degradation is increased, as proteolytic enzymes are secreted and activated in the paracellular spaces surrounding activated cells.

Transmigration of Phagocytic Cells

Following their development from stem cells in bone marrow, newly differentiated PMNs and monocytes enter the bloodstream and circulate throughout the body. On receiving a priming stimulus, they leave the blood by transmigration across the endothelium of postcapillary venules. Cell surface molecules belonging to several families of adhesion proteins and chemokine receptors regulate the transmigration of PMNs and monocytes.^{74,75} The first phase of the transmigration process involves margination, a passive process related to the relative slowness of blood flow in postcapillary venules, and the active process of rolling along the vascular endothelial surface.⁷⁵

Rolling is under the control of selectins and their counterreceptors.^{75,76} L selectins of leukocytes and E and P selectins of endothelial cells form binding contacts with appropriate carbohydrate-rich cell surface ligands.^{74,77} The selectin transmembrane proteins are concentrated at the tips of leukocyte and endothelial microvilli.⁷⁵ During the formation of these contacts, the leukocyte is slowly transported, in a rolling fashion, along the surface of the endothelium. Rapid proteolytic cleavage and shedding of the selectins allows the leukocyte to roll along the endothelial surface. Tumor necrosis factor α stimulates selectin-mediated capture and transmigration of leukocytes.⁷⁸

During the rolling phase, leukocytes come into contact with chemokines bound to the endothelial cell surface (discussed in chapter 13). Endothelial cell surface proteoglycans trap cytokines secreted locally by the endothelium or by other cells in the adjacent tissue. Interleukin 8 is the major chemokine acting on PMNs, and MCP-1 is the prime chemokine activator of monocytes and macrophages.⁷⁹ The signaling pathways activated during chemokine stimulation trigger an increase in the binding affinities of integrins to their extracellular matrix ligands, and they induce a migratory polarity of the leukocyte toward the basal lamina and underlying connective tissue.

Chemokine-induced signaling leads to the formation of stronger bonds between leukocyte integrins and their ligands.⁸⁰ These adhesions stop further rolling of the leukocyte. Plasma membrane integrins exist in either a low-affinity or a high-affinity ligand-binding state. The high-affinity mode is acquired following a conformational change in the integrin molecule (activation), triggered during chemokine activation of the leukocyte. This is an example of in-

tegrin inside-out signaling following a receptor-ligand interaction.

The $\beta 2$ and $\beta 1$ classes of integrins expressed on leukocytes form attachments to adhesion proteins of the immunoglobulin superfamily, including intercellular adhesion molecule 1 (ICAM-1), vascular adhesion molecule 1, and platelet-endothelial cell adhesion molecule 1, expressed on endothelial cells^{17,18,48,74,75,78,81} (see Fig 13-18 for a listing of potential adhesive pairings). Intercellular adhesion molecule 1 is expressed constitutively on endothelial cells, while platelet-endothelial cell adhesion molecule 1 and endothelial leukocyte adhesion molecule are expressed during an inflammatory response. The local concentration of cytokines and chemokines increases the adhesion and transmigration of leukocytes across the endothelium.

Laser scanning confocal microscopy and fluorescent antibodies have made it possible to view the localization of various adhesion molecules and cytoskeletal proteins during leukocyte transmigration across an endothelial cell layer.⁹ In the process of transmigration, neutrophils and other mononuclear leukocytes cross the endothelium by creating a circular transmigration passage between adjacent endothelial cells. A leukocyte leading-edge cell process (pseudopod) rich in LFA-1, F-actin, and catenins interacts with the endothelial cell surface in forming this channel.⁹

The endothelial zonula adherens junction must undergo localized disassembly and rapid reassembly during the transmigration of the leukocyte. Evidence suggests that there may be a cadherin-mediated interaction between the transmigrating leukocyte and the endothelial cells, preserving the adherens contact. Endothelial cell actin and myosin rearrangements and myosin light chain phosphorylation are induced by contact with transmigrating neutrophils, indicating that the endothelial cell is not a passive bystander during leukocyte transmigration.⁸²

Once past the endothelium, the migrating cell must breach the basement lamina, utilizing the proteolytic action of elastase and matrix metalloproteinases.^{34,83} Transepithelial migration of PMNs through the junctional epithelium also involves cell-to-cell adhesive interactions. In comparison to transendothelial migration, much less is known about the mechanism of leukocyte migration through epithelial barriers. Research studies have shown that the ability of an epithelium to support PMN transmigration is directly proportional to its capacity to generate IL-8. In vitro studies of PMN transmigration through intestinal epithelium have demonstrated the importance of PMN integrin (CD11b-Cd18) and epithelial CD47, a

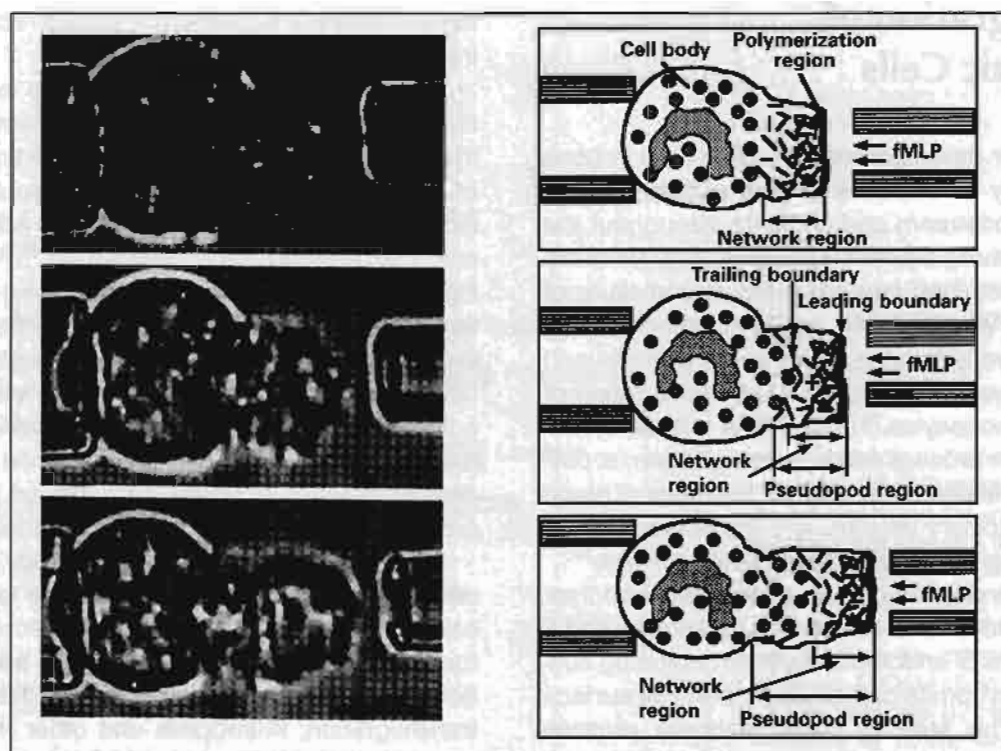


Fig 14-6 Formation of a polymorphonuclear neutrophil pseudopod and F-actin network in response to chemotactic factor *N*-formyl-methionyl-leucyl-phenylalanine (fMLP). The actin polymerization region is located beneath the plasma membrane at the leading edge. It gives rise to a network of actin filaments that supports the extension of the pseudopod. As the process advances, granules diffuse toward the trailing boundary. (Adapted from Zhelev et al⁹⁵ with permission from John Wiley & Sons.)

cell surface glycoprotein.^{84,85} Because adhesions and de-adhesions are needed for migration, it has been suggested that CD47 may act to deregulate integrin-mediated adhesive contacts between leukocytes and epithelial cells.⁸⁴

Chemotaxis

Polymorphonuclear neutrophils respond to a number of chemoattractant substances, of which IL-1 β , IL-8, LPS, C3a, C5a, leukotriene B₄, TNF- α , GM-CSF, and platelet-derived growth factor are among the most potent.^{1,86-88} Both β 1 and β 2 integrins expressed on PMNs, monocytes, and macrophages participate in transmigration and chemotaxis.^{17,18,62,89} Chemotaxins and chemokines increase the display of integrins by stimulating the transport of storage vesicles to the plasma membrane, and they "activate" the integrin molecules by inducing conformational changes.

The β 2 integrins (LFA-1 [CD11a-CD18, α L β 2] and Mac-1 [CD11b-CD18, α M β 2]), which bind to ICAM-1, are involved in the transmigration phase of PMN and monocyte chemotaxis.^{62,90} Once phagocytic cells enter the connective tissues, the β 1 integrins and their major counterreceptors, fibronectin and collagen, assume greater significance in chemotaxis.^{23,91} Fibronectin fragments containing arginine-glycine-aspartic acid sequences can block chemotaxis of PMNs mediated by IL-8. This finding supports the notion that integrin-fibronectin interaction is required for neutrophil migration.⁹²

Monocyte chemoattractant protein 1, a member of the Cys-Cys (CC) chemokine family, is a potent attractant for monocytes.⁹³ Monocyte chemoattractant protein 1 is produced by several cell types in response to stimulation by IL-1, IL-2, TNF- α , and basic fibroblast growth factor.⁹⁴

In a series of delicate experiments involving the stimulation of single neutrophils with micropipettes

filled with fMLP, investigators have been able to observe the polymerization of F-actin networks beneath the region of the plasma membrane containing the fMLP-binding sites.⁹⁵ These studies reveal a dynamic localized polymerization and depolymerization of F-actin networks during pseudopodal extension toward a chemoattractant stimulus.

As the cell engages the chemoattractant stimulus, a narrow zone of F-actin polymerization develops just underneath the cell surface in contact with the chemoattractant (Fig 14-6). A band of trapped actin filaments forms between the cytoplasm of the cell body and the leading edge of the pseudopodal extension. Secretory granules and vesicles are excluded from this region by the density of the F-actin network. At the border between the zone of newly polymerized F-actin and the cytoplasm, there is depolymerization of actin filaments, presumably generating G-actin used to supply the growing F-actin filaments at the polymerization front.

The width of the trapped F-actin network, about 2 μm , remains relatively stable during the entire phase of pseudopod extension. It was calculated that the turnover time of F-actin is about 20 seconds and that the rate of pseudopod extension is proportional to the concentration of chemoattractant or to the number of occupied receptors.⁹⁵ During a chemotactic response an elevation of cytoplasmic pressure is generated by a slight increase in the tension of the cortical cytoplasmic filament network. It has been proposed that this rise in pressure drives cytosolic fluid into the growing pseudopodal extension.

Phagocytosis

Neutrophils and macrophages are specialized for phagocytosis, the ingestion of particulate materials, such as bacteria, or fragments of dead cells and enclosure of the materials within cytoplasmic vacuoles or phagosomes (Figs 14-7 and 14-8). Lytic enzymes and free radicals are subsequently added to phagosomes through the fusion of lysosomal granules (specific and azurophilic granules in the case of PMNs). The progressive fusion of lysosomal granules with phagosomes leads to degranulation.

Phagocytosis and degranulation are coordinated by complex signaling cascades regulated by numerous inflammatory mediators. Phagocytosis is accompanied by a burst of oxidative metabolism for the production of ROMs. During phagocytic activation, proton-pumping enzymes are inserted into the plasma membrane and phagosome membranes

through fusion and exocytosis of vesicles and tertiary granules.⁹⁶ The H^+ -adenosine triphosphatase (proton pump) also buffers the rise in metabolic acid associated with cell activation by extruding hydrogen ions from the cytosol into the extracellular space and phagosomes.

The first step in phagocytosis is a recognition event carried out by the binding of cell receptors to ligands on the target particle. The receptor-ligand interaction may be direct, in which case receptors on the phagocyte bind directly to molecular components of the target. Phagocytosis may be aided by opsonins, intermediary molecules such as complement and immunoglobulins that form a coating over the surface of the target, in a process called *opsonization*.

Immunoglobulin G antibodies specific for antigens on a bacterial cell may act as opsonins, forming a coat over the bacterial surface (see Fig 14-8). Receptors on the phagocyte for the Fc portion of the antibody molecules bind the antibodies in a zipper-like process that is coordinated with plasma membrane flow around the target. When the target has been fully circumscribed, the plasma membrane fuses, and a phagosome is formed. Engagement of the Fc receptors also activates signaling pathways for granule transport and cytokine release.⁹⁷

In complement opsonization, the target is coated first with complement fragments (C3b). Recognition then occurs through binding to plasma membrane complement receptors CR2 and CR3 (CD11b-CD18 integrin complex). During the priming of PMNs by proinflammatory cytokines, the expression of integrins, including CR3, is upregulated, and their binding affinity for ligands is increased in preparation for phagocytosis.

Phagocytic cells also bind and phagocytose microorganisms using nonopsonic mechanisms involving integrins and pattern recognition molecules.⁶³ Pattern recognition receptors expressed on PMNs and macrophages, such as scavenger receptors and mannose receptors, bind a wide variety of negatively charged ligands, including the lipoteichoic acids of Gram-positive bacteria, low-density lipoproteins, polysaccharides, LPSs, collagen, and negatively charged particulate matter.^{17,98-101}

Other transmembrane receptors that have linkages to the cytoskeleton, such as CD44 and $\beta 1$ integrins, participate in the phagocytosis of cell debris and bacteria.^{58,72,102} Fibronectin-coated bacteria are readily phagocytosed by macrophages. Fibronectin binding to integrin receptors on macrophages potentiates phagocytosis.⁷² Macrophages also secrete

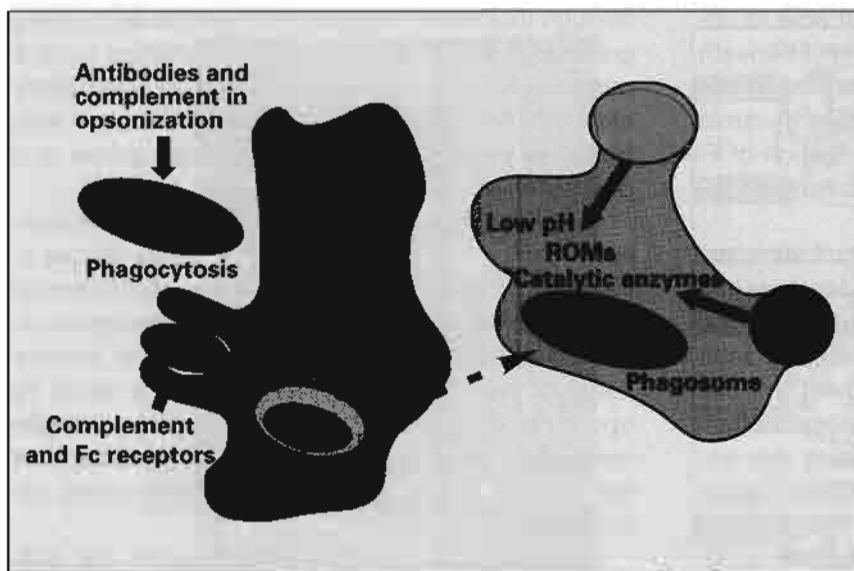


Fig 14-7 Formation and degranulation of phagosomes. During phagocytosis, azurophilic granules and secondary granules are transported to phagosomes. Following membrane fusion, the catalytic and bactericidal contents are released into the phagosome, where they come into direct contact with the invading organisms. The low internal pH activates enzymes and facilitates the production of reactive oxygen metabolites (ROMs).

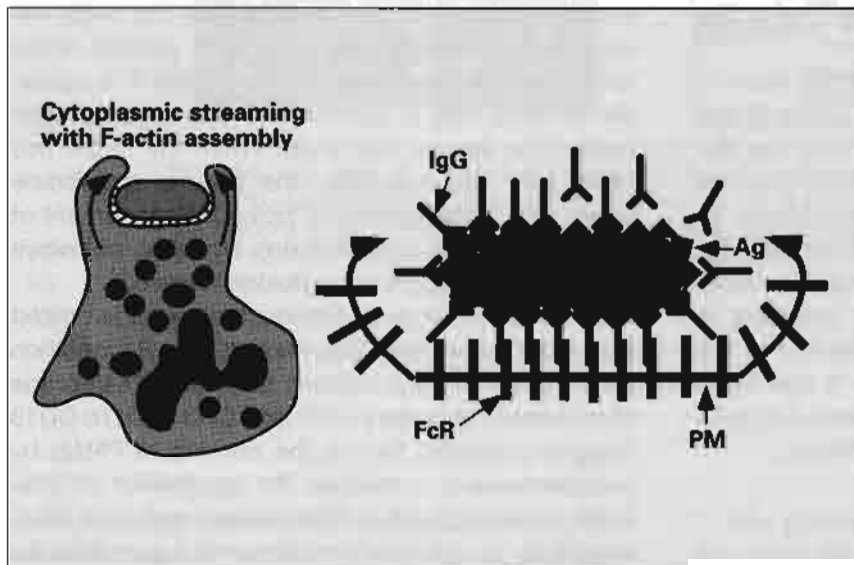


Fig 14-8 Opsonization. Immunoglobulin G (IgG) molecules attached to bacterial surface antigen (Ag) react in zipperlike binding to polymorphonuclear neutrophil plasma membrane (PM) Fc receptors (FcR). Similar opsonization reactions occur via C3b bound to bacterial surfaces and polymorphonuclear neutrophil complement receptor 3, and via membrane-bound mannan-binding protein with mannan-rich bacterial or viral carbohydrates.

osteopontin and appear to phagocytose osteopontin-coated calcified debris, most likely through integrin receptors for osteopontin.¹⁰³ Macrophages help to protect against systemic endotoxic shock by binding LPS on scavenger receptors.¹⁰⁴

Whether the initial attachment is mediated by opsonic or nonopsonic interaction, the immediate effect is to activate signaling cascades that recruit F-actin polymerization nuclei to the cell surface adjacent to the activated receptors. Receptor-ligand signaling during phagocytosis involves activation of tyrosine kinases, similar in many respects to activation of the B-cell receptor and T-cell receptor. The best-studied opsonin receptors, the FcR molecules,

contain ITAMs on the cytoplasmic carboxy terminals.¹⁰⁵ Following ligand binding, the ITAM units are phosphorylated by Src family cytoplasmic tyrosine kinases. Once ITAMs are phosphorylated, they recruit other cytoplasmic tyrosine kinases, which in turn activate the actin polymerization machinery. In order to allow cytoplasmic shape changes, and to provide G-actin monomers, existing F-actin filaments in adjacent cytoplasm must undergo depolymerization. The increase in gelsolin activity observed during phagocytosis is indicative of heightened turnover in the actin filament network.

The events of phagocytosis of single and multiple zymosan particles by neutrophils have been studied

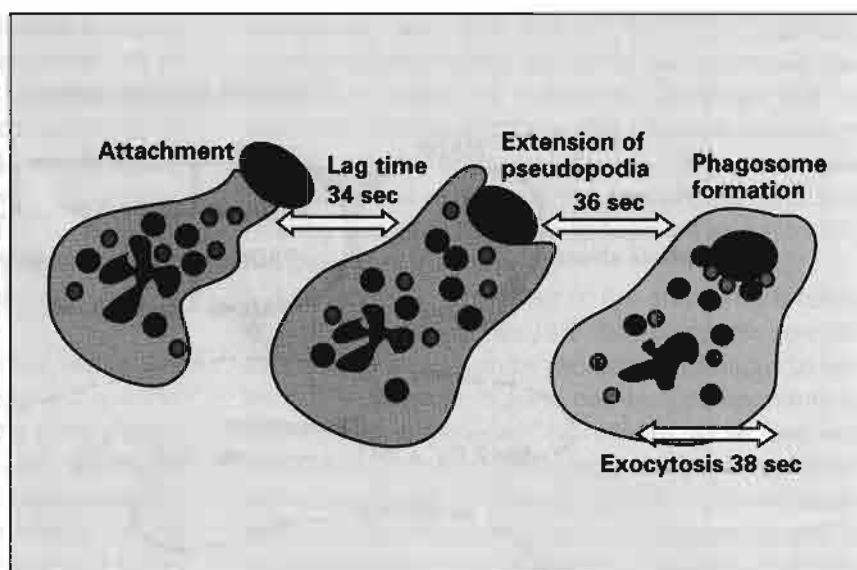


Fig 14-9 Timed sequential events in the formation of a phagosome as detected by high-resolution videocinematic photography. (Adapted from Susaki et al¹⁰⁸ with permission from John Wiley & Sons.)

with high-resolution videomicroscopy to record the time sequence of particle attachment, pseudopod formation, phagosome completion, and granule exocytosis. During phagosome formation, cytosolic calcium concentration is elevated, and elements of the cytoskeleton undergo activation. Phagocytosis and granule exocytosis into the phagosome compartment occur in a matter of about 90 seconds.

The first step involves contact between a short cytoplasmic extension and the zymosan particle, followed by a 34-second lag before the emergence of pseudopods (Fig 14-9).¹⁰⁶ After an additional 36 seconds, the particle is completely engulfed by the neutrophil. Granule exocytosis begins prior to the merger of the cytoplasmic pseudopods and continues for about 38 seconds. The merger and fusion of granules, visualized by interference videomicroscopy, takes place very rapidly. Thirty to forty granules fuse with a single phagosome.¹⁰⁶ Individual PMNs can form multiple phagosomes sequentially; the first to form receives the highest input of granules.

Macrophages and related cells of the reticuloendothelial system play an important role in disposing of apoptotic cells, such as effete red blood cells, lymphocytes, and PMNs.^{58,107} These cells undergo programmed cell death in large numbers as a normal physiologic consequence of their rapid turnover. Macrophages have been described as the "garbage men" of the body. It might be more appropriate to call them the "morticians" of the body. A dramatic example

of the efficiency of phagocytosis of apoptotic cells occurs in the thymus. Approximately 95% of all T cells entering the thymus are digested inside macrophages as part of the process of eliminating self-reacting T cells.

One aspect of apoptosis is a loss of lipid asymmetry in the plasma membrane.¹⁰⁸ Phosphatidylserine, normally restricted to the internal aspect of the lipid bilayer, migrates to the external layer, where it acts as a recognition signal for macrophages.¹⁰⁹ Macrophages also use scavenger receptors, lectin, lipid, and integrin-mediated recognition systems to identify dying cells.^{107,110} The macrophage vitronectin receptor has been shown to be involved in the recognition of apoptotic cells.¹¹¹

Recent studies have uncovered a potential role for macrophages in downregulating inflammation by inducing neutrophil apoptosis. During phagocytosis of apoptotic PMNs, macrophages release soluble Fas ligand that induces further apoptosis in bystander PMNs and monocytes, thereby accelerating the attenuation of inflammation.¹¹²

Generation of Reactive Oxygen Metabolites

The ability of granulocytes and macrophages to kill invading microorganisms is in large degree due to their ability to generate ROMs.^{31,113-115} Bacterial chemoattractant substance fMLP, platelet-activating factor,

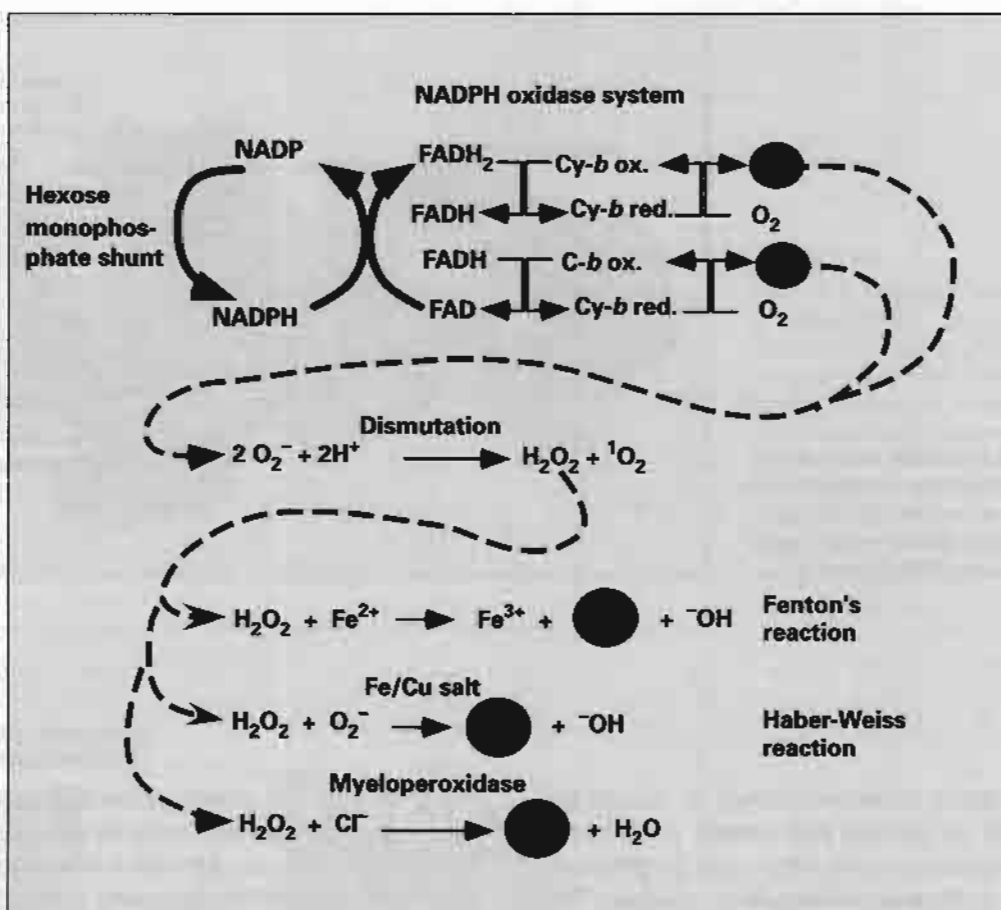


Fig 14-10 Pathways in the generation of reactive oxygen metabolites. Components of the oxidase system generate superoxide anion (O_2^-) from oxygen. In the acidic milieu of the phagosome, hydrogen peroxide (H_2O_2) is formed by the dismutation reaction. Hydrogen peroxide enters several reactions catalyzed by halide ions to generate hydroxyl free radicals ($\cdot OH$). (Cy-b ox.) Cytochrome *b* oxidase; (Cy-b red.) cytochrome *b* reductase; (FAD) flavin adenine dinucleotide; (FADH, FADH₂) reduced forms of FAD; (NADP) nicotinamide adenine dinucleotide phosphate; (NADPH) reduced form of NADP.

leukotriene B₄, immunoglobulin G immune complexes, and opsonized bacteria are among the more potent activators of ROM production. The highly toxic nature of ROMs resides in their ability to react with and destroy proteins in their immediate vicinity.

An unwanted side effect of the activation of the oxidase pathway is nearby tissue and cell damage.¹¹⁶ Contact between PMNs and opsonized surfaces, between PMNs and aggregated immunoglobulin-antigen complexes, triggers a rapid rise in the utilization of oxygen (respiratory burst) and the production of ROMs. Receptor binding of chemoattractant molecules also activates a respiratory burst, in addition to triggering cytoskeletal changes required for cell motility.¹¹⁷

The rapid rise in the utilization of oxygen is the result of the activation of the components of the oxidase system, which includes nicotinamide adenine dinucleotide phosphate reduced form (NADPH), cytochrome *b*, and flavin adenine dinucleotide.^{31,115} To avoid damaging side effects from premature and nonspecific activation of ROMs, the components of the oxidase system responsible for generating free radicals must remain unassembled and nonfunctional until an appropriate stimulatory receptor-ligand interaction occurs at the cell surface.¹¹⁸

Components of the oxidase system are stored in the cytosol and the limiting membranes of specific granules, gelatinase-containing granules, and secretory vesicles. Following receptor-ligand activation,

granules and components of the oxidase complex are translocated to the plasma membrane or to phagosomes.¹¹⁸⁻¹²⁰ Phosphokinase C migrates to the plasma membrane, where it regulates assembly of the oxidase complex.¹²¹ The oxidase system is then assembled to form an electron transport chain for the transfer of electrons from NADPH to O_2 , generating superoxide (O_2^-) in the process (Fig 14-10). Cytosolic components of the system include several GTP-binding proteins and NADPH.

The generation of superoxide occurs within the phagocytic vacuole following the fusion of specific granules to the limiting membrane of the phagosome. During the generation of superoxide, the supply of NADPH is replenished via the hexose monophosphate shunt pathway. The acidic pH of the interior of the phagosome favors the conversion of superoxide to hydrogen peroxide (H_2O_2) via spontaneous dismutation carried out by superoxide dismutase (see Fig 14-10). Hydrogen peroxide is in turn converted into the hydroxyl free radical ($\cdot OH$).^{31,115} This highly reactive compound destroys bacteria and a wide variety of biologically active molecules.

The bactericidal action of H_2O_2 is boosted by myeloperoxidase, an enzyme contained in azurophilic granules. Myeloperoxidase converts H_2O_2 and Cl^- to $HOCl$ and H_2O .^{31,115} Hypochlorous acid ($HOCl$) is a highly reactive product capable of killing bacteria by destroying the ion permeability barrier of bacterial membranes.³¹ Reactive oxygen metabolites have been shown to activate latent collagenase released by activated PMNs and thereby initiate degradation of extracellular matrix.³⁴

Antimicrobial Agents of Phagocytes

The destruction of microbes within the phagosomes of neutrophils takes place by a combination of oxidative and nonoxidative mechanisms. With the invagination of the plasma membrane to form a phagosome, the antimicrobial compounds of the primary and secondary granules are released into the newly formed phagosome. Among the chief actors in the nonoxidative killing pathway are the defensins, serine proteases, acid, lysozyme, and calprotectin.¹²²

The defensins are small cationic peptides with antimicrobial and cytotoxic activities. They are also known as *human neutrophil proteins*.¹²² They kill by inserting themselves into membranes to form aqueous channels.¹²³ Permeabilized bacterial membranes permit entry of other noxious agents such as ROMs,

cathepsins, and acid that kill the microorganism. Gram-positive bacteria and fungi are especially susceptible to attack by defensins. Defensins that escape from activated phagocytes have the capacity to bind C1q and thereby activate complement. Gingival crevicular fluid contains high levels of defensins, suggesting that they may have a function in controlling the periodontal microbiota.¹²⁴

Cathepsin G, a member of the granzyme B family of serine proteases, stored in the azurophilic granule, exerts its killing action by proteolysis of bacterial proteins.¹²³ Lysozyme digests cell wall components of Gram-positive bacteria. Calprotectin, a calcium- and zinc-binding protein contained in high concentration in the neutrophil cytoplasm, exerts a microbistatic action on bacteria and fungi in cytoplasm as well as in extracellular fluids. During the formation of ROMs, protons are generated and pumped into the phagosome to reduce the pH to below 6. The acidic environment of the phagosome may damage some bacteria directly and others through the activation of proteases.

The major components of the oxidase system include proteins of the NADPH complex, superoxide dismutase, and myeloperoxidase. The end products of the activation of the oxidase system are hydrogen peroxide, hydroxyl free radicals, and hypochlorous acid, all cooperating to kill bacteria within the phagosome.³¹ Although neutrophils can kill microorganisms without oxygen, the production of ROMs potentiates nonoxidative killing mechanisms.

Role of Phagocytes in Regulating Inflammation

In addition to their phagocytic function, PMNs and monocytes-macrophages regulate inflammation and immune responses through the release of proinflammatory mediators such as IL-1, IL-6, IL-8, IFN- γ , TNF- α , granulocyte colony-stimulating factor, GM-CSF, and eicosanoids. They also produce inhibitory regulators of cytokine activity, such as cytokine receptor antagonists (IL-1 receptor antagonist) and soluble cytokine-binding proteins (soluble TNF receptor and soluble IL-1 receptor).¹²⁵

Neutrophils, monocytes, and macrophages are an important source of eicosanoids, a class of short-acting metabolites derived from the enzymatic alteration of cell membrane phospholipid fatty acids. The eicosanoids include prostaglandins, thromboxanes, leukotrienes, and lipoxins. Phospholipase A2 cleaves membrane fatty acids to produce arachidonic acid, a

major precursor of eicosanoid synthesis. Prostaglandins and thromboxanes are produced in the cyclooxygenase pathway, while the leukotrienes are generated in the lipoxygenase pathway. The prostaglandins exert a wide range of actions; some promote inflammation while others have an inhibiting effect. The actions of leukotrienes and thromboxanes are mostly proinflammatory.

Cytokines and prostaglandins have long been recognized as playing significant roles in the pathogenesis of gingivitis and periodontal disease.¹²⁵⁻¹²⁷ They increase in gingival crevicular fluid along with the development of gingivitis and are present in increasing levels in the gingival crevicular fluid of patients with periodontitis.¹²⁵⁻¹²⁷

Aggregation of Polymorphonuclear Neutrophils

Activation of PMNs increases integrin expression and binding affinity, thereby increasing adhesiveness and aggregation. As a result of their increased adhesiveness, PMNs often aggregate in layers to "wall off" an area of infection. Presumably the ability to limit intercellular permeability between adjacent PMNs acts as a protective barrier. In leukocyte adhesion deficiency disease, peripheral blood PMNs fail to aggregate when activated.

When PMNs attach to a large activating surface, or to an opsonized particle that is too large to be phagocytosed, PMNs aggregate and lysosomal granules are transported to the cell surface abutting the activating surface. During this process, the lysosomal granules and ROMs are released into the extracellular space. In this "frustrated phagocytosis" there is a potential for tissue damage because of the leakage of lysosomal enzymes and free radicals. However, the increased adhesiveness of activated PMNs tends to obliterate the lateral intercellular space and to minimize a backward flow of toxic substances.

The interaction of gingival PMNs and dental plaque provides an informative biologic example of the tendency of activated PMNs to wall off an area of infection.¹²⁸

Cytokine Regulation of Phagocytic Cells

Interleukin 10 has a potent anti-inflammatory action because of its ability to downregulate IL-1, IL-8, TNF- α , and GM-CSF in T_H1 cells. Interleukin 10 shortens

the longevity of PMNs by increasing the rate of apoptosis.¹³ It also decreases phagocytosis by PMNs and monocytes by decreasing $\beta 2$ integrins.¹²⁹ The $\beta 2$ integrins mediate nonopsonized phagocytosis of bacterial cells.

Under the influence of IL-10, macrophages and monocytes secrete tissue inhibitor of matrix metalloproteinase 1 and decrease secretion of 92-kDa gelatinase.¹³⁰ Another action of IL-10 is its ability to increase IL-1 receptor antagonist, thereby reducing the proinflammatory effect of IL-1 on PMNs, monocytes, and macrophages. Interleukin 10 also decreases osteoclast formation by its negative regulation of osteoclast precursors.¹³¹

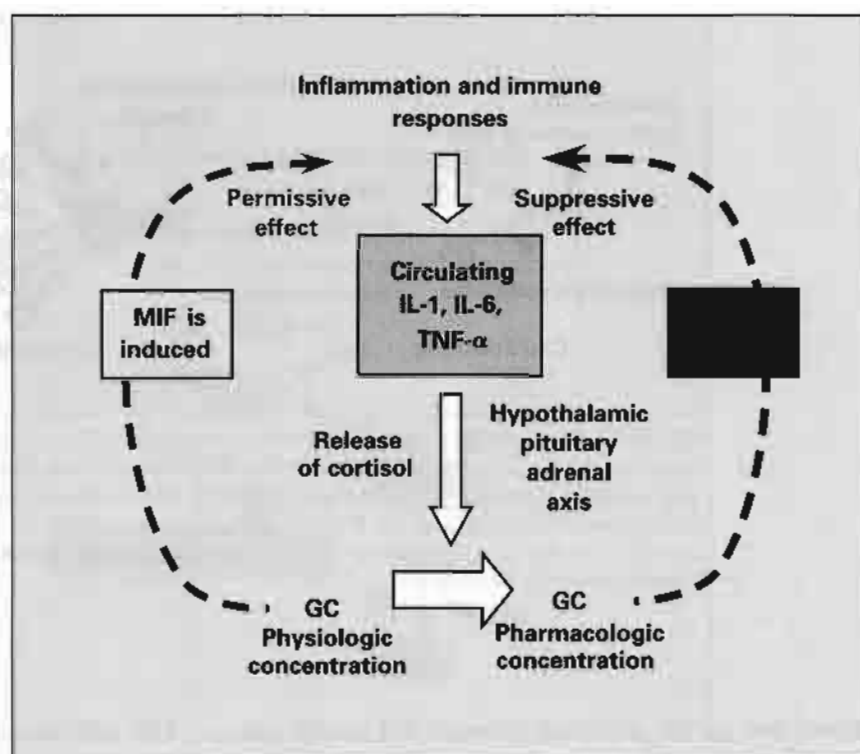
Interleukin 4 also has an anti-inflammatory effect.¹³² It blocks the spontaneous and LPS-stimulated production of proinflammatory cytokines in monocytes and reduces macrophage collagenase, ROM production, and prostaglandin synthesis.¹³³ Interleukin 4 inhibits release of IL-8 by PMNs. By downregulating CD14 and the Fc receptors, it minimizes LPS- and antibody-dependent inflammatory pathways. However, IL-4 increases dendritic cell differentiation and promotes phagocytosis.¹³²

The mediators IL-1, IL-6, IL-12, GM-CSF, IFN- γ , and TNF- α activate monocytes and PMNs and prolong their life by postponing apoptosis.¹³⁴ These substances also stimulate phagocytosis by PMNs and macrophages.¹³⁵ Tumor necrosis factor α and IFN- γ act synergistically to prolong the inflammatory response.¹³⁶

Various members of the chemokine family are potent activators of phagocytic cell migration. Interleukin 8 (a CXC chemokine) promotes chemotaxis of neutrophils, while several CC chemokines, such as MCP-1 and RANTES (regulated on activation, normal T cell expressed and secreted), induce migration of monocytes and dendritic cells.¹³⁷⁻¹³⁹ The importance of IL-8 in inflammation is underscored by the demonstrated ability of anti-IL-8 antibodies to decrease the severity of several inflammatory diseases.¹⁴⁰

Both CXC and CC chemokines are produced in increased amounts by mononuclear cells when challenged by periodontal pathogens.¹⁴¹ Of related interest is the ability of TNF- α and IFN- γ to induce the production of IL-8 and RANTES in oral keratinocytes, suggesting a potential participation of oral epithelial cells in creating chemotactic gradients for neutrophils and mononuclear phagocytes.¹⁴² Human periodontal ligament cells treated with IL-1 β express increased amounts of IL-6 and decreased levels of IL-10, providing support for the view that resident cells can amplify the inflammatory response during periodontal disease.¹⁴³

Fig 14-11 Inflammation and immune responses. Glucocorticoids (GC) at physiologic concentrations have a permissive action on many aspects of the inflammatory process. This action is mediated by macrophage migration inhibitory factor (MIF). At high concentrations of GC (induced by circulating cytokines: interleukin 1 [IL-1], interleukin 6 [IL-6], and tumor necrosis factor α [TNF- α]), MIF is inhibited, resulting in an immunosuppressive action.



Macrophage migration inhibition factor (MIF), present in unstimulated monocytes and macrophages, is released on stimulation by proinflammatory stimuli, including LPS and TNF- α . Migration inhibition factor attracts monocytes and macrophages and induces them to release proinflammatory cytokines, such as TNF- α , IL-1, IL-6, and IL-8, thereby creating a local amplification loop in peripheral tissues¹⁴⁴⁻¹⁴⁶ (Fig 14-11). Of related interest is the recent discovery that MIF is produced by pituitary cells and is secreted into the bloodstream, where it acts systemically in opposition to the immunosuppressive effects of glucocorticoid hormones,^{144,145} which are discussed in more detail later in this chapter.

Structure and Function of the Complement System

Several extracellular enzyme systems mediate key biologic responses of acute inflammation, wound repair, and host defense against viral and bacterial challenges. These extracellular enzymes work hand-in-hand with granulocytes and with cells of the im-

mune system to help protect the host. There are four major systems:

1. Complement
2. Kinins
3. Coagulation factors
4. Fibrinolytic factors

Each system involves sequential activation of extracellular enzymes. Many of the resulting cleavage products act as biologic mediators of cellular function.

The complement system will be discussed because of its importance in the oral defensive system and its potential involvement in disease pathogenesis. The complement system is made up of a family of more than 30 serum proteins that perform potent accessory functions in immune responses and during inflammation. The complement system evolved in invertebrates some 700 million years ago, long before the appearance of the adaptive (antibody) system.¹⁴⁷

The three major activities of the complement system are the opsonization of bacteria and antibody complexes, the activation of phagocytic cells and B lymphocytes, and the lysis of target cells. Comple-

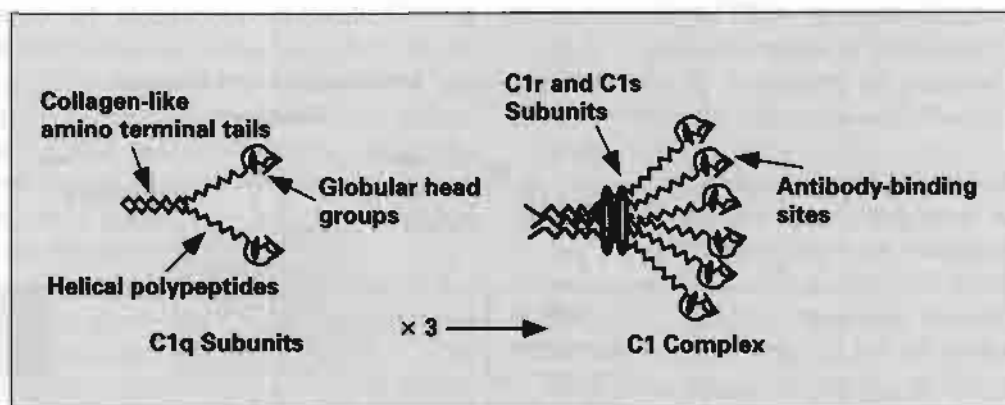


Fig 14-12 Components of the C1q complement molecule. The C1 component of complement is made up of C1q, C1r, and C1s proteins. The C1q polypeptide contains a collagen-like domain, a middle helical segment, and a globular head group. Two C1q polypeptides assemble to form a subunit with an antibody-binding site. Each C1 complex contains three C1q subunits and two additional proteins, C1r and C1s. The latter is a serine protease activated by conformational changes in C1q following antibody binding.

ment also serves as a link between the innate and adaptive immune systems through its ability to engage coreceptors during B-cell activation.

Activation

Complement is made primarily in the liver, but other cell types such as keratinocytes, macrophages, fibroblasts, and endothelial cells also contribute to the production of complement proteins.¹⁴⁸ The proteins of the complement system, most of which are proenzymes, must undergo cleavage to generate biologically active subunits. Some of the cleavage products act as ligands for specific complement receptors found on several cell types, including macrophages, lymphocytes, and PMNs.

The most abundant and the key component of complement is a protein called C3. It is cleaved by the enzyme C3 convertase, thereby producing C3a and C3b.¹⁴⁹ Of the two products, C3b is the primary effector molecule, capable of binding to bacteria and antigen-antibody complexes.

There are two major pathways for activating the enzyme C3 convertase and generating C3b: the classic pathway, activated by antigen-antibody complexes, and the alternative pathway, activated by complex carbohydrates found on bacterial or other type of surfaces. The classic pathway is adaptive, in that it follows immune recognition of nonself, while the alternative pathway represents an innate, more primitive, nonspecific defense system.^{147,149} A third pathway of complement activation is the lectin pathway.

This pathway is activated by a variety of plasma proteins (such as mannose-binding protein) that bind microbial polysaccharides and subsequently activate the C1 component of complement.¹⁵⁰

Classic pathway

Activation of the classic pathway begins with the interaction of the C1 complex with antigen-antibody complexes or with antibody-coated surfaces. The C1q molecule is made up of three subunits, each consisting of two globular head groups connected to collagen-like amino terminals, via midregion helical domains (Fig 14-12). Assembly and stabilization of the three subunits to form C1 requires calcium and two C1r and two C1s polypeptides positioned astride and below the globular head groups. In the classic pathway of complement activation, C1q serine esterase (C1s) is activated following binding of antibody-antigen complexes via the interaction of the globular head groups to the Fc portions of the antibody molecules.

On binding to appropriate counterligands, C1q undergoes conformational changes. These changes activate C1s serine esterase activity (Fig 14-13). This enzyme functions at two points in the activation cascade: it acts to convert the complement proteins C4 and C2 into C4bC2 and subsequently generates C3 convertase (C4b2a). The latter enzyme complex converts C3 to C3a and C3b, a precursor of the important biologic mediator C5a. As a result of the slow hydrolysis of C3, a small amount of active C3b is always present in biologic fluids.

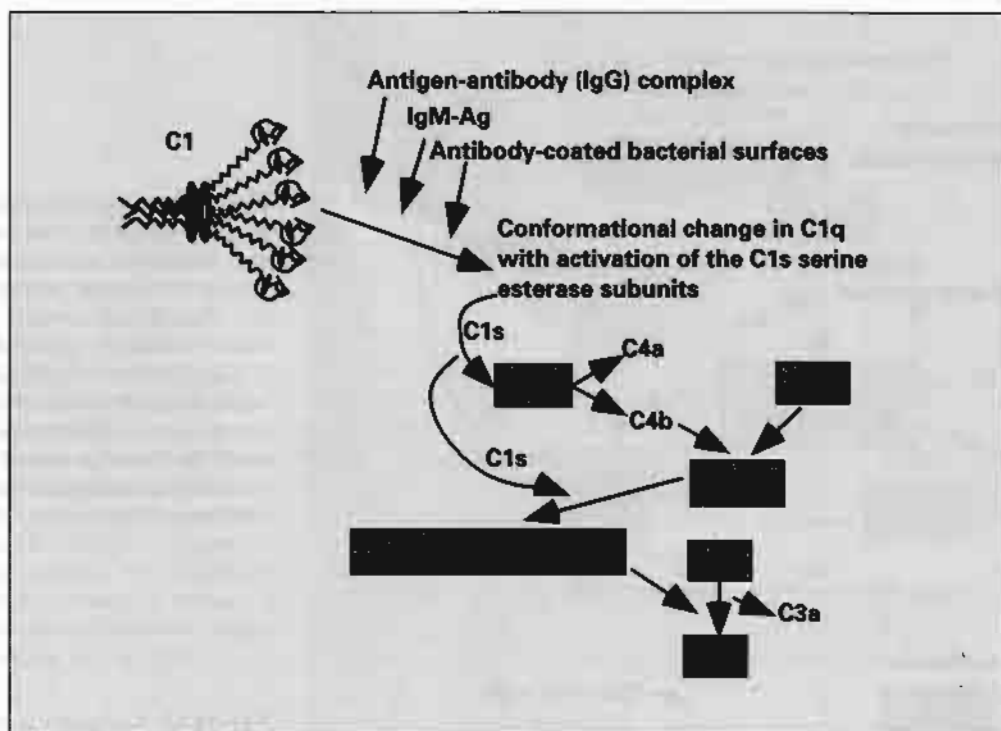


Fig 14-13 Classic pathway of complement activation. The C1 complex interacts with antibody to initiate the activation of C1s serine esterase. It splits C4, generating C4b, which binds to C2, forming a substrate for additional C1s action. C4bC2 is converted to C4b2a (C3 convertase) by C1s. C3 convertase splits C3 into C3a (potent chemotxin) and C3b (opsonin, phagocyte activator, and proximal component for the alternative pathway and the membrane attack complex). (Ag) Antigen; (IgG) immunoglobulin G; (IgM) immunoglobulin M.

Alternative pathway

In the alternative pathway, active C3b forms a complex with factor B on various surfaces (Fig 14-14). Bacterial surfaces facilitate the formation of these complexes. Through the action of factor D, a serine esterase present in extracellular fluids, C3bFB is converted to C3bBb, another form of C3 convertase. The cascade of complement activation in the alternative pathway is amplified through formation of more C3b from C3 (see Fig 14-13). Furthermore, C3bBb plus C3b may subsequently interact to form C3bBb3b (C5 convertase) (Fig 14-15).¹⁶

Lectin pathway

Complement is also activated through the interaction of mannan-binding protein to mannose-rich surfaces (Fig 14-16). In this lectin pathway a mannan-binding protein-associated serine esterase cleaves C3 and C4, generating C3b and C4b.

Membrane attack complex

One of the primary functions of the complement system is the formation of a membrane attack complex designed to cause lysis of target cells and bacteria.^{149,151} Formation of a membrane attack complex is started when C5 is cleaved by C5 convertase, which is generated in the classic pathway and the alternative pathway when C3b is bound by one of the C3 convertase enzymes (C4b2a or C3bBb) (see Fig 14-15). Two C5 cleavage products are generated, C5a and C5b. The latter molecule initiates a nonenzymatic assembly process, starting with C6 and C7, to form a hydrophobic complex that associates with cell membranes. Subsequently, C8 attaches to the complex and serves as a nidus for the assembly of several C9 molecules to form a water-filled channel through the plasma membrane. Creation of aqueous channels leads to ionic imbalances, cytoplasmic swelling, and cell death.

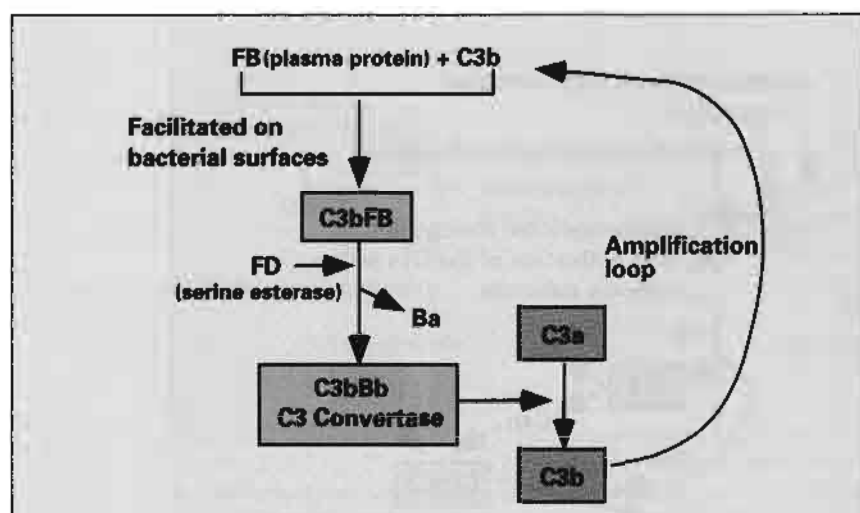


Fig 14-14 Alternative pathway of complement activation. The alternative pathway is initiated following the binding of C3b and a plasma protein, factor B (FB), to a "foreign" surface such as a bacterial outer membrane. C3bFB is split into C3bBb (a C3 convertase) and a smaller fraction (Ba) by factor D (FD), a serum serine esterase. C3bBb splits C3 into C3b and C3a. Newly generated C3b may bind to activating surfaces, amplifying complement activation.

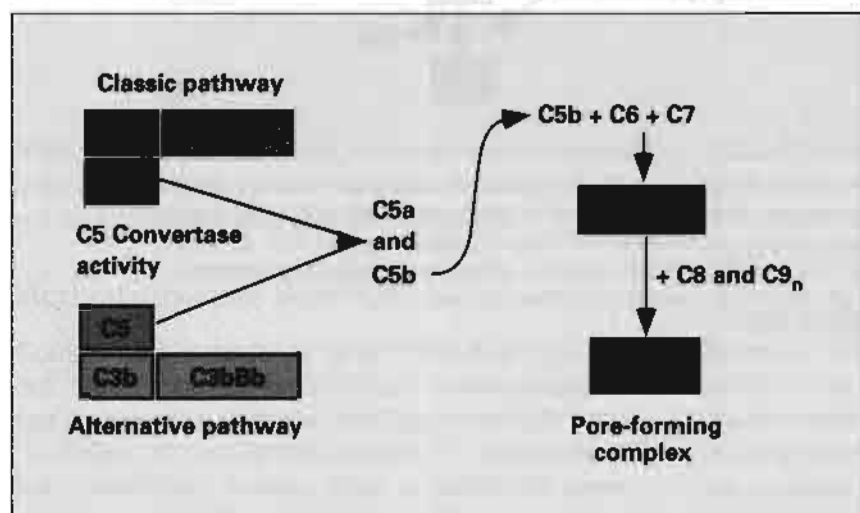


Fig 14-15 Formation of the membrane attack complex. The membrane attack complex requires the sequential assembly of C5b, C6, C7, C8, and C9 complement proteins at the target cell membrane. The cascade is started by the formation of C5 convertase, requiring C3b and C3 convertase, generated in either the classic or the alternative pathways. C5 is split into C5a and C5b. C5b binds C6 and C7 to form C5b67, a complex at the membrane. It subsequently recruits C8 and multiple molecules of C9 to form a pore in the target membrane.

Oponization and coreceptor functions

Additional functions of the complement system, such as opsonization and coreceptor stimulation, require the association of complement molecules with specific cell surface receptors on monocytes-macrophages, dendritic cells, endothelial cells, and PMNs.¹⁶ Complement receptor 1 ([CR1] CD35) binds C3b and C4b. Bacteria, which have been opsonized by a coating of C3b, are phagocytosed following their attachment to CR1 molecules on phagocytic cells (Fig 14-17).

Complement receptor 2 ([CR2] CD21) is located on B lymphocytes, follicular dendritic cells, and ep-

ithelial cells.^{152,153} Both CR2 and complement receptor 3 (CR3) bind IFN- γ and C3. When associated with its ligands, CR2 potentiates the activation of B cells by acting as a coreceptor with B-cell receptor.¹⁵⁴ Complement receptors on follicular dendritic cells have been shown to be important for antigen trapping and initiating costimulation of B lymphocytes.¹⁵³

Complement receptors 3 (CD11b-CD18) and 4 (CD11c-CD18) are members of the $\beta 2$ integrin family. They are present on PMNs, macrophages, natural killer cells, and antigen-presenting cells, where they act as binding sites for C3 cleavage products. Both CR3 and CR4 appear to function in the phagocytosis of targets opsonized by C3b.

Fig 14-16 Lectin pathway of complement activation. Mannose-rich and *N*-acetylglucosamine-rich surfaces can activate complement by binding serum-derived mannan-binding protein (MBP) and the MBP-associated serine esterase (MASP). In this pathway, MASP cleaves C3 and C4, generating C3b and C4b, respectively. Each of the two products goes on to react with other complement components to generate C3 convertase activity. The pathway on the left resembles the alternative pathway, while the one on the right resembles the classic pathway. The MBP-MASP pathway, or lectin pathway, is believed to be a primitive innate defensive system used against viruses and bacteria.

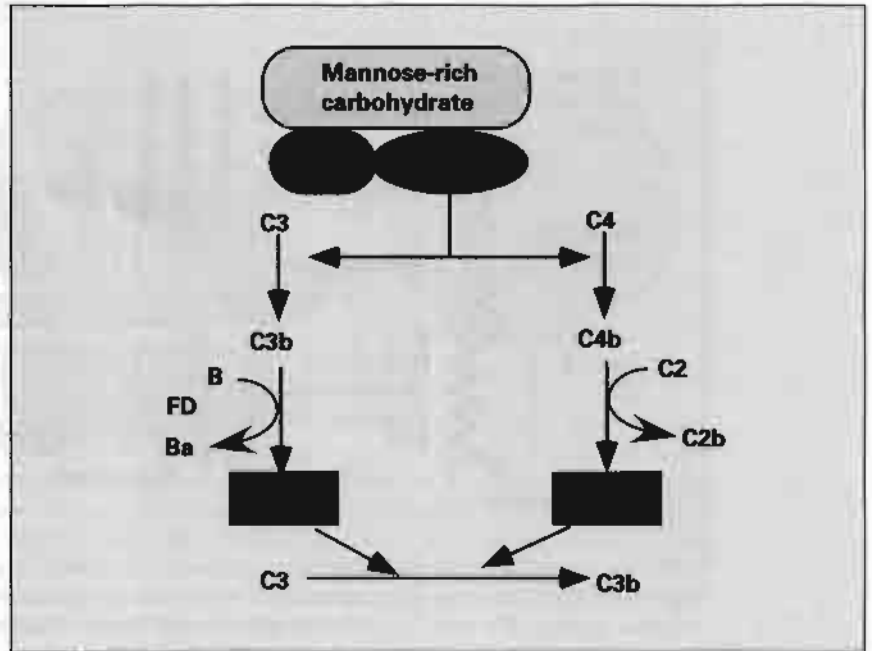
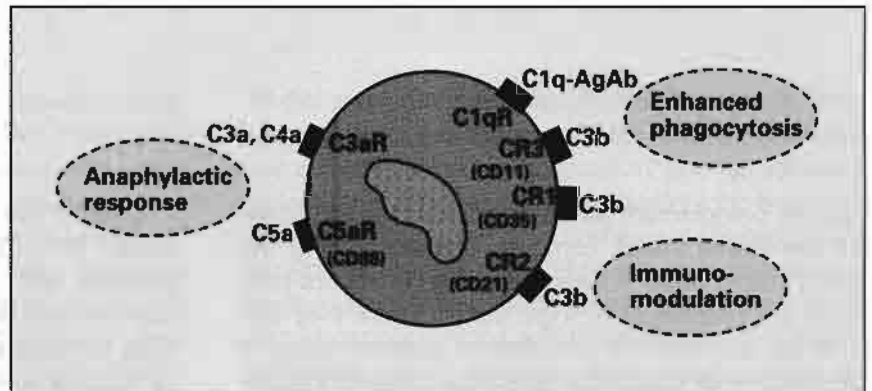


Fig 14-17 General responses elicited following complement-receptor engagement. Phagocytosis is stimulated by opsonization following binding of C1q-antigen-antibody (C1q-AgAb) complexes to the C1q receptor (C1qR). Engagement by C3b of the receptor CR3 also increases phagocytosis. Receptors CR1 and CR2 bind C3b, facilitating B-cell activation. Receptors for C3a and C5a activate acute proinflammatory (anaphylactic) reactions, such as mast cell degranulation and polymorphonuclear neutrophil chemotaxis.



Anaphylatoxins

Two small complement cleavage products, C3a and C5a (also known as *anaphylatoxins*), have key biologic effects during inflammation. Complement receptors C3aR and C5aR are G protein-linked, seven-pass transmembrane receptors located on many cell types, including phagocytes.¹⁶ The products C3a and C5a cause rapid degranulation of mast cells, initiate PMN chemotaxis and granule discharge, and increase vascular permeability (see Fig 14-17). These results are not always beneficial to the host, especially if activated on a large scale. Bacterial LPS is a powerful activator of complement (alternative pathway), generating C3a and C5a and in turn increasing the exudation and degranulation of PMNs.

Progress in understanding the structure of C5a and C5aR has led to the development of potent C5aR antagonists. These molecules were produced by modification of the effector C-terminal domain of C5a.¹⁵⁵ These C5aR antagonists block PMN integrin upregulation, superoxide generation, chemotaxis, and lysozyme release.¹⁵⁵

Complement control mechanisms

To minimize the inappropriate activation of complement and the destruction of host cells, several control mechanisms have evolved to regulate the system.¹⁴⁹ Soluble inhibitors and cell membrane inhibitory proteins accomplish regulation. Soluble inhibitors include vitronectin and clusterin. Both proteins bind

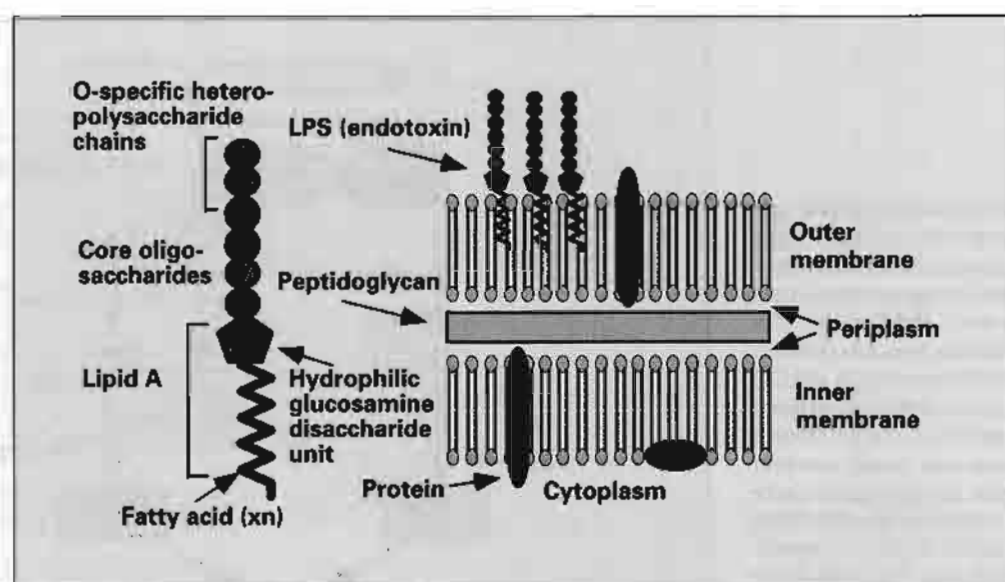


Fig 14-18 Structure of lipopolysaccharide (LPS) and its position in the outer membrane of Gram-negative bacteria. The toxic properties of the molecule reside in the lipid A portion. The outer segments of the LPS molecule are species specific. Lipopolysaccharide is released along with portions of the outer membrane in the form of small vesicles.

soluble complement products to block the formation of membrane attack complex, thereby limiting the lysis of host cells.¹⁴⁹ Serine esterase inhibitors (serpins) are present in biologic fluids, where they can limit the activation of C1s. Serum proteins (factor 1 and C4b-binding protein) catabolize C4b and thereby limit the formation of C3 convertase.

Cell membrane inhibitors include protectin (CD59), a membrane-linked protein that blocks the formation of membrane attack complex by preventing the incorporation of C9.¹⁴⁹ An example of the importance of CD59 is provided by recent studies that show that defective CD59 on neurons can lead to assembly of membrane attack complex and complement-mediated lysis in Alzheimer's disease.¹⁵⁶ Protectin is strongly expressed on healthy gingival epithelial cells, where it may limit epithelial cell lysis.¹⁵⁷ Other membrane inhibitory molecules include decay-accelerating factor (CD55) and CR1 (CD35).¹⁴⁹ Both molecules inhibit binding of C2 to C4b to form C4b2a (C3 convertase) and accelerate its disassociation. Endothelial cells express high levels of cell surface decay-accelerating factor and deposit it in the subepithelial extracellular matrix as an additional protection against vascular activation of complement.¹⁵⁸

Despite the aforementioned safeguards, there is always the potential for aberrant activation of complement with serious consequences for the host. Complement degradation fragments can activate host cells to release biologically active substances such as histamine, reactive oxygen metabolites, vascular permeability factors, and lysosomal enzymes. The complex nature of complement-fragment-induced responses and tissue injury has been reviewed recently.¹⁵⁹

Biologic Effects of Lipopolysaccharide

Gram-negative bacteria produce endotoxins that elicit inflammatory reactions and polyclonal immune responses. Lipopolysaccharides, a component of the outer membrane of Gram-negative bacteria, are responsible for the endotoxin effect. Lipopolysaccharide is a protein-free molecule subdivided into a lipid A domain, a core oligosaccharide, and a cluster of species-specific heteropolysaccharide sidechains (Fig 14-18).¹⁶⁰ The lipid A components contain several fatty acid chains covalently linked to a hydrophilic glucosamine disaccharide unit.

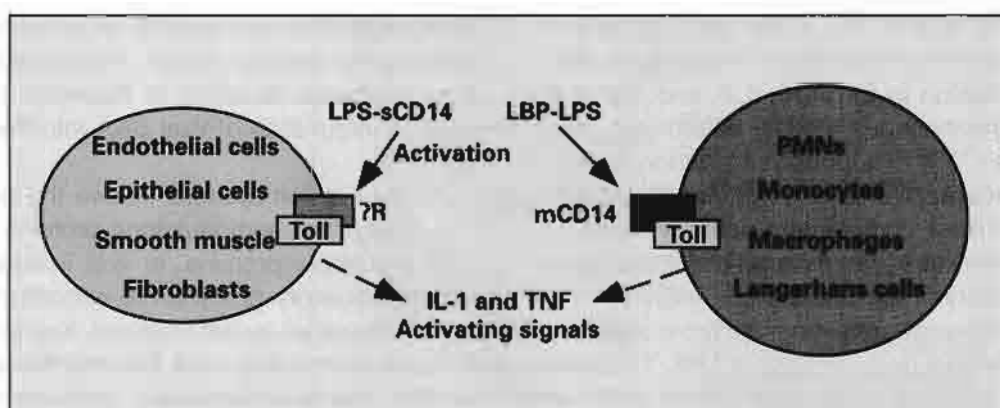


Fig 14-19 Cellular activation by lipopolysaccharide (LPS). Lipopolysaccharides bind to LPS-binding protein (LBP) or to soluble CD14 (sCD14) in the extracellular fluids. The LPS-sCD14 complex subsequently binds to an unidentified cell membrane receptor (?R) to activate several cell types, including endothelial cells and fibroblasts. LBP-LPS complexes bind to cell membrane CD14 (mCD14) on polymorphonuclear neutrophils (PMNs), monocytes, macrophages, and Langerhans cells. Both signaling pathways appear to require a newly identified family of Toll-like membrane receptors. Activation of these cells leads to the production of interleukin 1 (IL-1) and tumor necrosis factor (TNF), which aid in the activation of cells that do not contain LPS receptors.

Lipopolysaccharide activates a wide variety of cells, including periodontal ligament fibroblasts, monocytes, and PMNs^{161,162} (Fig 14-19). Lipopolysaccharide exerts its effect by interacting with CD14, a pattern recognition molecule, and with a serum LPS-binding protein.¹⁹ There are two forms of CD14: a plasma membrane component (mCD14) and a soluble molecule (sCD14) in serum and tissue fluid. Polymorphonuclear neutrophils, monocytes, and macrophages bind LBP-LPS through mCD14, while cells that do not have mCD14 use a yet-to-be-identified receptor to bind sCD14-LPS complexes¹⁶³ (see Fig 14-17).

Because CD14 is a peripheral membrane protein, activation following LPS binding to mCD14 requires participation of a recently identified Toll-like receptor, CD14-associated signaling protein.¹⁶⁴ Lipopolysaccharide can also activate PMNs via CD14 without the need for LPS-binding protein if the LPS is bound to a substrate such as the surface of a microbe or on collagen.¹⁶⁵ Activation of CD14 also has a role in phagocytosis and in T-cell activation. Monocytes can phagocytose Gram-negative bacteria by a CD14-dependent mechanism without engagement of Fc receptors or integrins.¹⁶⁶ Activation of T lymphocytes by LPS is CD14 dependent but MHC independent. It requires costimulation from monocytes via CD28-B7 signaling.¹⁶⁷

One of the earliest effects of LPS is on PMNs. It causes a rapid rise in integrin-mediated adhesion of

PMNs and increases emigration of PMNs from blood vessels. Prior exposure of PMNs to LPS potentiates the activation of phospholipase A2 and the synthesis of leukotrienes in response to chemotactic factors.¹⁶⁸ Lipopolysaccharide-CD14 complexes formed on the plasma membrane of the PMN are endocytosed, triggering signal transduction pathways that lead to protein phosphorylations and the activation of mitogen-activated protein kinases. As a result, genes are activated for the synthesis of TNF- α , IL-1, IL-6, and IL-8.¹⁶⁹ Oral bacterial LPS also induces prostaglandin E₂ production by peripheral blood monocytes with potential systemic consequences.¹⁷⁰

Gingival fibroblasts express ICAM-1 in increasing amounts following activation by binding sCD14-LPS complexes.¹⁷¹ Increased levels of ICAM-1 may promote increased retention and interaction of various inflammatory cells with gingival fibroblasts. Lipopolysaccharides from several periopathogenic bacteria activate interleukins and cytokine release from gingival fibroblasts.^{162,172,173}

Gram-negative bacteria in plaque release LPS, usually in the form of small vesicles. These substances are absorbed in the gingival tissues and the adjacent cementum. Clear evidence that bacterial LPS in dental plaque gains access to host tissue is provided by the presence of plasma cells with LPS-specific antibody in the gingival lamina propria of patients with periodontal disease.

The potential role of LPS in the pathogenesis of periodontal destruction stems from its ability to stimulate the expression of IL-1, IL-6, IL-8, and TNF- α in monocytes, macrophages, PMNs, endothelial cells, and fibroblasts.^{161,173-175} Lipopolysaccharide also induces polyclonal activation of lymphocytes and activates the alternative complement pathway. Bone resorption may be increased through the osteoclastic stimulating activity of IL-1 and IL-6.¹⁷³

Serum contains natural antibodies (immunoglobulin M) that provide a defense against LPS. These antibodies bind LPS and help to clear it from the circulation.¹⁵²

Glucocorticoid Modulation of the Inflammatory Response

Physiologic concentrations of glucocorticoids are needed for the development of a normal inflammatory-immune response. At high concentrations, however, glucocorticoids exert an anti-inflammatory effect. This bimodal action is explained in part by the control glucocorticoids have over the expression of macrophage MIF¹⁴⁶ (see Fig 14-11). Since its discovery as an inhibitor of macrophage migration, this cytokine has been shown to have a broad range of proinflammatory actions, including upregulation of macrophage function, TNF- α expression, and T-cell activation. At low (physiologic) concentrations glucocorticoids induce MIF, while at high (pharmacologic) concentrations they inhibit MIF activity¹⁴⁶ (see Fig 14-11).

During inflammation, IL-1, IL-6, and TNF- α are released into the systemic circulation. They act on the hypothalamic-pituitary-adrenal axis to increase the production of MIF by cells of the pituitary gland and the adrenal medulla. Macrophages are also a major local source of MIF.

High levels of glucocorticoids have numerous anti-inflammatory actions, including suppression of activation of T cells and other effector cells, inhibition of the production of proinflammatory cytokines, and inhibition of the expression of adhesion factors.

Immunomodulatory Evasion Mechanisms of Microbes

In the course of evolution, bacteria and viruses acquired ways to avoid the immune systems of the host. The development of these characteristics gave certain microorganisms a selective advantage by improving their chances of survival and proliferation.

Microorganisms use passive or active mechanisms to evade the immune system. Passive evasion ranges from antigenic variation of microbial surface antigens to integration of viral DNA into the genome of the host.

Active evasion systems involve the production of microbial immunomodulatory proteins that mimic host regulatory proteins. In viral infections, the immunomodulatory proteins are encoded by the virus and synthesized by the host cell. Key aspects of the immune system that have become the targets of microbial immunomodulatory proteins include the complement system, cytokine expression, cytokine regulatory signaling pathways, antigen processing and presentation, and apoptosis (Fig 14-20).¹⁷⁶⁻¹⁷⁸

The ability to produce molecules that mimic complement proteins has been discovered in viruses, bacteria, fungi, and various parasites. Some large double-stranded DNA viruses encode a complement control protein (VCP) that binds C3 and C4, thereby blocking the complement system's ability to recruit mononuclear cells. With fewer mononuclear cells responding to the infectious agent, fewer cytokines are produced and the defensive reaction is minimized. Secretory immunomodulatory proteins such as VCP are called *virokines*.

Viroceptors form another class of virus-encoded secretory immunomodulatory proteins (Fig 14-21). These proteins mimic host receptors for cytokines or other types of regulatory molecules. The viroceptor proteins are homologous to the extracellular domain of normal transmembrane receptors. Over the course of time, viruses that incorporated only the DNA segment that encoded the binding domain of the receptor protein gained selective advantage. The viroceptor proteins lacking cytoplasmic and transmembrane domains become secreted into the pericellular fluid, where they are free to bind cytokines.¹⁷⁸ An example of a viroceptor evasion is the ability of vaccinia virus to encode an IL-1 β receptor-like molecule that is released from infected cells. Other forms of cytokine receptor mimicry involve TNF, IFN- γ and IL-8.

Certain viruses have evolved methods to avoid detection within the host cell by virtue of their ability to interfere with antigen processing and presentation. Herpesviruses block the transport of antigenic peptides into the endoplasmic reticulum by encoding a protein that binds to the adenosine triphosphate binding cassette transporter. Thus, the MHC-I molecules fail to load antigenic peptides and are not transported to the cell surface. Human immunodeficiency virus also has evolved a mechanism for decreasing the level of MHC-I expression in infected cells.

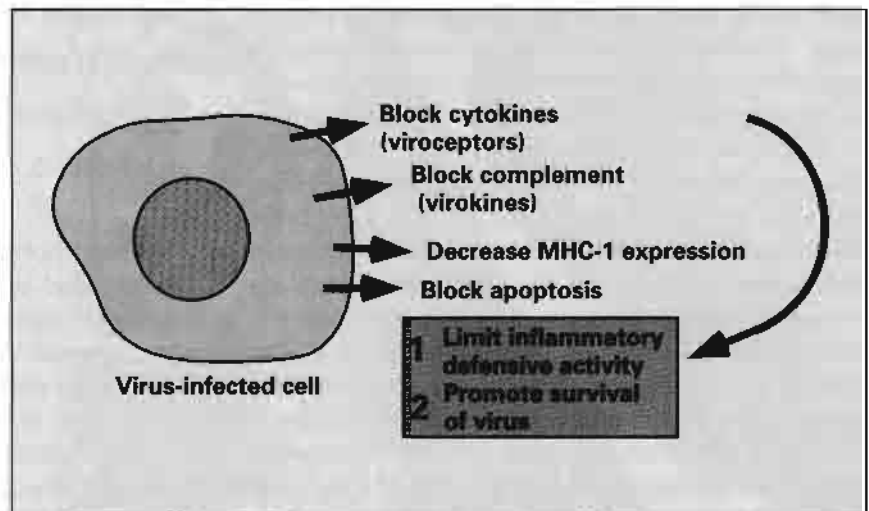


Fig 14-20 Various mechanisms used by viruses to limit host defenses and to increase their chances of survival. (MHC-I) Major histocompatibility complex type I.

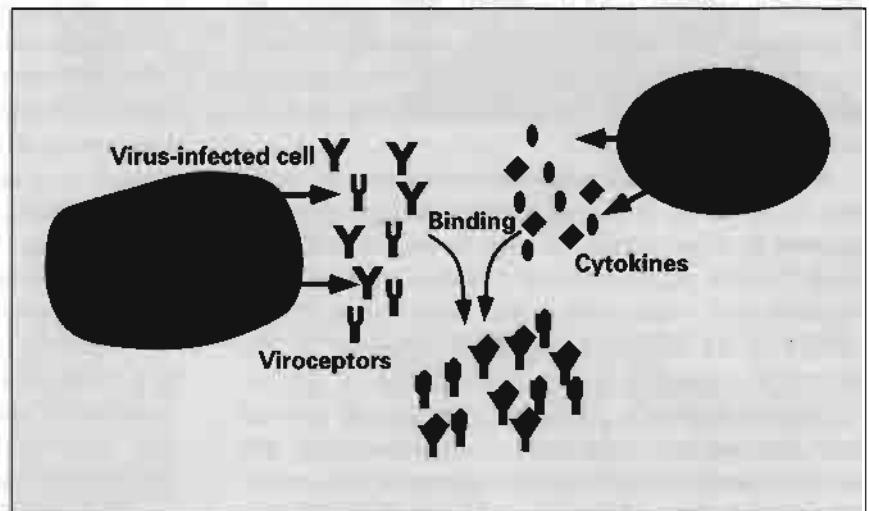


Fig 14-21 Viroceptor inactivation of cytokines. Certain viruses can encode receptor-like proteins that have no transmembrane anchoring domains. These viroceptors are released into the extracellular space, where they can bind cytokines that have been produced locally by cells of the immune system. In this way, the viruses limit the number of cytokine molecules available to stimulate and regulate the local inflammatory response. (Adapted from Kotwal¹⁷⁸ with permission.)

Some viruses evade the immune system by blocking the destruction of the infected cell by cytotoxic T cells. The Epstein-Barr virus induces the expression of a protein that mimics Bcl-2, a protein that interferes with TNF and Fas apoptotic pathways.

Some bacterial pathogens, such as *Borrelia burgdorferi*, the microbe that causes Lyme disease, avoid attack from complement and antibody-mediated defenses by invading living cells.¹⁷⁹ Certain microorganisms have evolved the ability to produce toxins that kill leukocytes. Leukotoxins produced by *Staphylococcus aureus* attack the neutrophil plasma membranes, creating pores that upset the ionic balance of the cytosol, leading to cell death and the discharge of inflammatory mediators and granule proteases.¹⁸⁰

Two microorganisms, *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis*, have been linked to the pathogenesis of periodontal disease. Both organisms have developed defensive systems that give them a survival advantage in the gingiva and in periodontal pockets.

Porphyromonas gingivalis microorganisms gain entry into gingival epithelial cells through a receptor-ligand zippering process involving the activity of epithelial cell cytoplasmic ruffles or microvilli.^{181,182} The natures of the bacterial ligand and the epithelial receptor are unknown. An intact epithelial cytoskeletal system and energy-consuming activities in both the cell and the bacterium are needed for internalization. A rapid rise in cytoplasmic calcium accompanies internalization. It is unclear if a vacuolar phase occurs

or if the bacteria escape into the cytoplasm immediately on internalization. *Porphyromonas gingivalis* multiplies inside the protected environment provided by the infected cell.^{182,183}

Porphyromonas gingivalis cysteine proteinases have been shown to be able to degrade tumor necrosis factor γ in vitro, suggesting that removal of proinflammatory mediators might be another mechanism that bacteria might use to evade the body's defenses.¹⁸⁴ *Porphyromonas gingivalis* cysteine protease (gingipain) cleaves C5a receptors on PMNs, thereby interfering with one arm of the neutrophil-complement-based antimicrobial defense system.¹⁸⁵ Epithelial cell cultures infected with *P. gingivalis* show decreased expression of IL-8 and MCP-1 when challenged by IL-1 α and TNF- α . Furthermore, PMN chemotaxis to fMLP through epithelial layers was decreased when the epithelial cells were infected with *P. gingivalis*. The capacity of *P. gingivalis* to decrease the expression of chemokines and the migration of neutrophils has the potential for increasing its periodontal pathogenicity.¹⁸³

Actinobacillus actinomycetemcomitans, in addition to its ability to kill neutrophils through the production of a leukotoxin, is able to invade epithelial cells.¹⁸⁶ Here again, the entry phase involves a receptor-ligand interaction; in this case, the epithelial receptors are known to be either integrins or the transferrin receptor. In the initial phase, *A. actinomycetemcomitans* is contained in a vacuole derived from the plasma membrane. Shortly thereafter, the bacteria break out of the limiting membrane, possibly through the activity of a phospholipase C enzyme, and gain access to the cytoplasm. *Actinobacillus actinomycetemcomitans* proliferates inside the cell before being released into the extracellular space or directly into an adjacent epithelial cell by the formation of a cytoplasmic bridge. Studies have shown that bacterial proliferation is accelerated inside the epithelial cytoplasm.

The ability of these two periodontal pathogens to gain entry into epithelial cells may protect them from neutrophils and allow them to proliferate in a sheltered environment. The combination of invasive activity with their potential for secreting various proteolytic enzymes makes them well suited to cause local tissue destruction.

Studies of *Streptococcus mutans* isolates from root surfaces of caries-free and caries-active subjects revealed that isolates from caries-active subjects were less able (by 50%) to activate PMNs, suggesting that they may have acquired a selective advantage by developing mechanisms for evading neutrophils.¹⁸⁷

Clinical Correlation: Polymorphonuclear Neutrophil Function and Periodontal Disease

The neutrophils are the most abundant inflammatory cells found in the gingival tissues and the gingival sulcus. They are present even in the gingiva of germ-free animals. Neutrophils form a key defensive barrier at the tooth-tissue interface (Figs 14-22a to 14-22c). The importance of PMNs in protecting the periodontal tissues is illustrated by the increased disease activity observed in patients with neutrophil deficiencies.

Three quarters of patients diagnosed to suffer from localized juvenile periodontitis have been found to have abnormalities in PMN chemotaxis. Localized juvenile periodontitis neutrophils have numerous defects in receptor expression and signaling events, suggesting that localized juvenile periodontitis is caused by a variety of genetic defects that affect the ability of PMNs to respond in a normal way to bacterial challenges.¹⁸⁸ Polymorphonuclear neutrophils from patients with localized juvenile periodontitis show decreased chemotaxis to C5a, IL-8, and fMLP.¹⁸⁹

In addition to fewer receptors for chemotactic factors, there are also abnormalities in the calcium and diacylglycerol second messenger responses to activation. Neutrophils of patients with localized juvenile periodontitis have been shown to have an elevated response to activation via Fc and complement receptor engagement.¹⁹⁰ Localized juvenile periodontitis is characterized by excessive bone resorption around the central incisors and molars, especially pronounced around puberty. Presumably the inability to destroy pathogenic organisms, such as *A. actinomycetemcomitans*, leads to increased concentrations of bone resorption promoters.

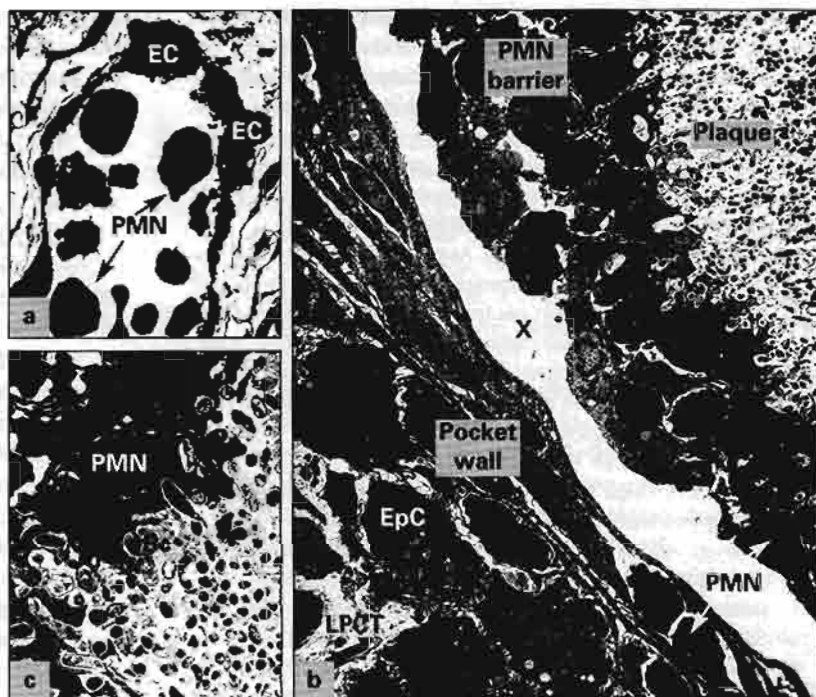
Leukocyte adhesion syndrome is a condition caused by faulty coding of the integrin (β 2, CD18) components of the PMN cell surface adhesion molecules LFA-1 (CD11a-CD18), Mac-1 (CD11b-CD18), and p150, p95 (CD11c-CD18).^{62,191,192} Emigration of PMNs from blood vessels, chemotaxis, and phagocytosis are compromised in these patients.^{189,193} As a result, they suffer from numerous systemic and local infections, including severe periodontal disease.⁴

Studies of gingival crevicular PMNs indicate that they are in the late stages of activation, judging by increased expression of surface integrins and complement receptors.^{194,195} Furthermore, there is evi-

Fig 14-22a Gingival venule containing polymorphonuclear neutrophils (PMN). The endothelial cells (EC) have a high cytoplasmic volume. (Original magnification $\times 1,500$.)

Fig 14-22b Low-magnification electron microscopic view of the polymorphonuclear neutrophil (PMN) barrier between the gingival pocket wall and plaque bacteria. (X) Artifactual space created during tissue fixation; (EpC) epithelial cells; (LPCT) lamina propria connective tissue. (Original magnification $\times 3,200$.)

Fig 14-22c Higher magnification of the polymorphonuclear neutrophil (PMN) barrier. At the outermost surface of the barrier, bacteria (Bc) are phagocytosed (arrows). (Original magnification $\times 5,000$.)



dence that the crevicular fluid PMNs of patients with periodontitis have higher amounts of integrins than do their counterparts in normal crevicular fluid.¹⁹⁶ Neutrophil enzymes, such as elastase-like serine proteases, collagenase, and cathepsin G, are increased in the crevicular fluid of patients with periodontitis.^{33,38}

Calprotectin, a leukocyte protein that may have antibacterial properties, is present in high concentration in gingival crevicular fluid.¹⁹⁷

Neutrophils are attracted to the gingival sulcus by a variety of chemotactic factors, among which IL-8 appears to be especially important. The LPS of *P. gingivalis* activates PMN synthesis of IL-8, thereby amplifying the inflammatory reaction by attracting new PMNs to the gingival crevice. Epithelial cells, fibroblasts, and neutrophils in patients with periodontitis produce IL-8. Periodontitis patients have elevated serum IL-8, and there is evidence that circulating neutrophils are activated.¹⁹⁸

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